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(54) Title: IMAGING INFECTION USING FLUORESCENT PROTEIN AS A MARKER

(57) Abstract: A method to follow the progress of infection in vertebrate subjects utilizes infective agents which have been modified to express a fluorescent protein. The method can also monitor expression of genes associated with infective agents during the course of infection. The method may further include targeting tumors with the modified infective agents.

## IMAGING INFECTION USING FLUORESCENT PROTEIN AS A MARKER

### Technical Field

5       [0001] The invention relates to the study of microbial and viral infection. Specifically, it concerns systems for studying progress of, and control of, infection in vertebrates and methods for evaluating candidate drugs and targeting tumors.

### Background Art

10       [0002] The use of green fluorescent protein to visualize cancer progression and metastasis is by now well established. See, for example, Hoffman, R.M., *Methods in Enzymology* (1999) 302:20-31 (P. Michael Conn, ed., Academic Press, San Diego). The use of whole body imaging to chart real time progression and to assess the efficacy of proposed protocols for treating tumors is disclosed in U.S. patent 6,251,384, the contents of which are incorporated herein by reference.

15       [0003] The advantages of green fluorescent protein have been noted in that it does not require any substrates or cofactors and its expression in living cells does not apparently cause any biological damage. In addition, the fluorescence emitted makes this a particularly sensitive technique. Indeed, the whole body images obtainable using simple equipment, *e.g.*, 490 nm excitation from a xenon or mercury lamp along with image capture by a CCD color video camera permit real-time investigations of tumor growth and metastasis. See, for example, Yang, M., *et al.*, *Proc. Natl. Acad. Sci. USA* (2000) 97:1206-1211.

20       [0004] The present invention extends the techniques developed in imaging tumor growth and metastasis to the study of infection. Microbial and viral infection can be monitored by labeling the infectious agent with a bright fluorescent protein and the progress of infection monitored. In addition, protocols useful in treating microbial or viral infection can be evaluated by taking advantage of this technique. The materials and methods for obtaining suitable  
25       expression of fluorescent proteins are readily available. For example, Cheng, L., *et al.*, *Gene Therapy* (1997) 4:1013-1022, describe the modification of hematopoietic stem cells with green fluorescent protein (GFP) encoding sequences under control of a retroviral promoter. Although the authors state that human stem cells are transfected with this system only with difficulty, by using an enhanced form of the GFP, satisfactory brightness could be achieved. Grignani, F., *et*

*al.*, *Cancer Res* (1998) 58:14-19, report the use of a hybrid EBV/retroviral vector expressing GFP to effect high-efficiency gene transfer into human hematopoietic progenitor cells.

[0005] Vectors containing various modified forms of GFP to provide various colors are marketed by Clontech. The Clontech vectors intended for mammalian cell expression place the GFP under control of the cytomegalovirus (CMV) promoter; such expression systems can also be used to label viral infectious agents.

[0006] Attempts have been made to visualize bacteria in mammalian subjects using luciferase as a marker, but because of the low luminosity of this system, whole body imaging is not practical. See, for example, Contag, P.R., *et al.*, *Nat. Med.* (1998) 4:245-247.

[0007] GFP expressing bacteria have been previously employed in a number of studies that were not in intact, living animals (Wu, H., *et al.*, *Microbiol.* (2000) 146:2481-2493; Ling, S.H.M., *et al.*, *Microbiol.* (2000) 146:7-19; Badger, J.L., *et al.*, *Mol. Microbiol.* (2000) 36(1):174-182; Kohler, R., *et al.*, *Mol. Gen. Genet.* (2000) 262:1060-1069; Valdivia, R.H., *et al.*, *Gene* (1996) 173:47-52; Valdivia, R.H., *et al.*, *Science* (1997) 277:2007-2011; Scott, K.P., *et al.*, *FEMS Microbiol. Ltrs.* (2000) 182:23-27; Prachaiyo, P., *et al.*, *J. Food Protect.* (2000) 63:427-433; Geoffroy, M-C., *Applied & Env. Microbiol.* (2000) 66:383-391). An example of such studies was the visualization of the *in vitro* infection of muscle tissue by the pathogenic *E. coli* O157H GFP (Prachaiyo, P., *et al.*, *supra*). Another approach examined the mouse gastrointestinal tract after gavage infection by removal and fixation of the gastrointestinal tissue (Geoffroy, M-C., *supra*). Fish infected with GFP transduced *Edwardsiella tarda* were imaged for infection after removal of their organs (Ling, S.H.M., *et al.*, *supra*). Genes associated with virulence and other infectious processes were evaluated by linkage to GFP expression (Ling, S.H.M., *et al.*, *supra*; Badger, J.L., *et al.*, *supra*; Kohler, R., *et al.*, *supra*; Valdivia, R.H., *et al.*, *supra* (1996).

[0008] The present invention also extends to targeting tumors to deliver therapeutics thereto via infective agents such as microorganisms using fluorescence. Attempts have been made to deliver the anaerobic bacteria *Clostridia novyi* to necrotic regions in tumors (Dang, L.H., *et al.*, *Proc. Natl. Acad. Sci. USA* (2001) 98:15155-15160). In addition, the necrotic regions of tumors have been targeted using *Bifidobacterium longum* (Yazawa, K., *et al.*, *Cancer Gene Therapy* (2000) 7(2):269-274 and Yazawa, K., *et al.*, *Breast Cancer Res. & Treatment* (2001) 66:165-170). These approaches depend on anaerobes, are targeted at necrotic tissue only and/or may be used only for tumors of a large size. Further, tumors have been targeted using

*Salmonella* that is devoid of its toxin (Low, K.B., *et al.*, *Nature Biotech.* (1999) 17:37-41). Additional studies have reported the tumor targeting capability of *Salmonella* in human patients with metastatic melanoma and renal cell carcinoma (Toso, J.F., *et al.*, *J. Clin. Oncol.* (2002) 20(1):142-152). These approaches do not provide a way to visualize the bacteria in living animals.

[0009] Bacteria and other microorganisms offer many features to deliver therapeutics to tumors. For example they are readily transformed to produce both human and specialized bacterial proteins. The bacterial proteins, however, include a wide variety and potency of toxins. In order to take advantage of such powerful molecules, it would be useful to have an accurate tumor-targeting mechanism for therapeutic-delivering bacteria as shown by the present invention.

#### Disclosure of the Invention

[0010] The invention provides models which permit the intimate study of formation of microbial or viral infection in a realistic and real-time setting. By using fluorescent proteins such as green fluorescent protein (GFP) as a stable and readily visualized marker, the progression of infection can be modeled and the mechanism elucidated. The invention is also directed, in part, to tumor targeting which depends on the ability to visualize the bacteria or microorganism as well as its therapeutic molecule.

[0011] Thus, in one aspect, the invention is directed to a method to monitor the course of infection in a model vertebrate system by monitoring the spatial and temporal progression of fluorescence in said vertebrate subject wherein said subject has been subjected to infection by a microbe or virus which microbe or virus expresses a fluorescent protein.

[0012] In another aspect, the invention is directed to a method to evaluate a candidate protocol or drug for inhibition of infection in a subject which method comprises administering the protocol or drug to a vertebrate subject which has been infected with a microbe or virus that expresses a fluorescent protein and monitoring the temporal and spatial progress of infection by observing the presence, absence or intensity of fluorescence at various locations at various times in the infected subject. In this method, in addition, the presence, absence or intensity of fluorescence at various locations in a control subject at various times is also monitored for comparison with the subject that has been treated with the protocol or drug. The progress of infection over time and space is compared in the treated subject and the control subject, and a



diminution of the intensity of infection in the treated subject as compared to the control subject identifies a successful protocol or drug.

[0013] In yet another aspect, the invention is directed to a method to target tumors using a therapeutic infective agent in a vertebrate subject comprising administering an infective agent that expresses a fluorescent protein to the vertebrate subject and observing the presence, absence, or intensity of fluorescence at various locations in the subject as a function of time. Preferably the therapeutic infective agent targets the tumor and delivers a therapeutic product to the tumor.

[0014] The methods of the invention can also be used to monitor the nature of the microbial or viral systems that are significant in the progress of infection by coupling the nucleotide sequence encoding the fluorescent protein to various positions in the genome of the microbe or virus and monitoring the expression of the fluorescent protein by monitoring the fluorescence.

[0015] Lastly, the invention is directed to a tumor-targeting infective agent that expresses a fluorescent protein that is capable of targeting tumors in intact, living mammals in comparison to normal cells.

#### Brief Description of the Drawings

[0016] Figures 1A-1H show the locations of fluorescence in various parts of a mouse administered  $10^{11}$  *E. coli*-GFP by gavage. Figure 1A shows evidence of infection in the stomach immediately after gavage; Figures 1B-1G show the presence of fluorescence in the small intestine 10, 20, 30, 40, 50 and 60 minutes after gavage, respectively. Figure 1H shows the presence of infection in the colon 120 minutes after gavage.

[0017] Figures 2A-2C show the results of intravital imaging of *E. coli* after gavage with  $10^{11}$  *E. coli*-GFP. As shown in Figure 2A, GFP infection is present in the stomach and the duodenum immediately after gavage; Figure 2B shows the presence of infection in the small intestine 40 minutes after gavage; Figure 2C shows the presence of infection in the colon 120 minutes after gavage.

[0018] Figures 3A-3B show whole body and intravital imaging of infection in the stomach, small intestine and colon after gavage. Figure 3A shows a whole body image in the stomach (arrowhead), small intestine (fine arrows), and colon (thick arrow) after multiple gavage of aliquots of  $3 \times 10^{11}$  *E. coli*-GFP. Figure 3B shows corresponding intravital images labeled similarly.

[0019] Figure 4 shows the results of whole body imaging of infection in the colon immediately after enema of  $10^{11}$  *E. coli*-GFP.

[0020] Figures 5A-5D show the results of whole body imaging of peritoneal cavity infection in antibiotic response. Figures 5A and 5C show the infection in the peritoneal cavity immediately after intraperitoneal (i.p.) injection of  $10^9$  *E. coli*-GFP. Figure 5B shows an untreated mouse six hours after injection; the animal died at six hours. Figure 5D shows a Kanamycin treated mouse six hours after i.p. injection, wherein the animal survived.

[0021] Figure 6 shows the results of intravital imaging of intraperitoneal infection as described in Figure 5.

[0022] Figure 7A shows whole body imaging of an RFP-labeled U-87 human glioma growing in a nude mouse. Figure 7B shows fluorescence-guided injection of a PBS solution containing GFP-labeled *Salmonella*. Figure 7C shows whole body imaging of a GFP-labeled *Salmonella* in the RFP-labeled U-87 human glioma immediately after injection. Figure 7D shows the GFP-labeled *Salmonella* growing in the RFP-labeled U-87 human glioma one day after injection.

[0023] Figure 8A shows whole body imaging of an RFP-labeled DU-145 human prostate tumor in a nude mouse (Mouse 1). Figure 8B shows GFP-labeled *Salmonella* injected in the tumor of Mouse 1 imaged immediately after injection. Figure 8C shows whole body imaging of an RFP-labeled DU-145 human prostate tumor in a nude mouse (Mouse 2). Figure 8D shows the results of GFP-labeled *Salmonella* injected in the RFP-labeled DU-145 human prostate tumor which was imaged immediately after injection in Mouse 2.

[0024] Figure 9A shows whole body imaging of an RFP-labeled MDA MB-435 human breast tumor growing in a nude mouse. Figure 9B shows whole body imaging of GFP-labeled *Salmonella* injected in the tumor immediately after injection.

[0025] Figure 10A shows whole body imaging of an RFP-labeled U-87 human glioma growing in a nude mouse. Figure 10B shows whole body imaging of a PBS solution containing GFP-labeled *Salmonella* injected in the glioma. Figure 10C shows whole body imaging of a GFP-labeled *Salmonella* in the RFP-labeled U-87 human glioma immediately after injection. Figure 10D shows whole body imaging of a GFP-labeled *Salmonella* growing in the RFP-labeled U-87 human glioma one day after injection.

[0026] Figure 11A shows whole body imaging of an RFP-labeled DU-145 human prostate tumor in a nude mouse (Mouse 1). Figure 11B shows the results of GFP-labeled *Salmonella*

injected in the RFP-labeled DU-145 human prostate tumor which was imaged immediately after injection in Mouse 1. Figure 11C shows whole body imaging of an RFP-labeled DU-145 human prostate tumor in a nude mouse (Mouse 2). Figure 11D shows the results of GFP-labeled *Salmonella* injected in the RFP-labeled DU-145 human prostate tumor which was imaged immediately after injection in Mouse 2.

[0027] Figure 12A shows whole body imaging of an RFP-labeled MDA MB-435 human breast tumor growing in a nude mouse. Figure 12B shows the results of GFP-labeled *Salmonella* injected in the tumor which was imaged immediately after injection.

[0028] Figure 13A shows whole body imaging of a GFP-labeled PC-3 human prostate tumor growing in a nude mouse. Figure 13B shows the results of RFP-labeled *Salmonella* injected in the tumor which was imaged immediately after injection. Figure 13C shows whole body imaging of an RFP-labeled *Salmonella* growing in the GFP-labeled PC-3 human prostate tumor one day after injection.

[0029] Figure 14A shows whole body imaging of a GFP-labeled PC-3 human prostate tumor growing in a nude mouse. Figure 14B shows the results of RFP-labeled *Salmonella* injected in the GFP-labeled PC-3 human prostate tumor immediately after injection. Figure 14C shows whole body imaging of an RFP-labeled *Salmonella* growing in the GFP-labeled PC-3 human prostate tumor one day after injection. Figure 14D shows whole body imaging of an RFP-labeled *Salmonella* growing in the GFP-labeled PC-3 human prostate tumor four days after injection.

[0030] Figure 15A shows whole body imaging of a GFP-labeled PC-3 human prostate tumor growing in a nude mouse. Figure 15B shows the results of RFP-labeled *Salmonella* injected in the GFP-labeled PC-3 human prostate tumor which was imaged immediately after injection. Figure 15C shows whole body imaging of an RFP-labeled *Salmonella* growing in the GFP-labeled PC-3 human prostate tumor one day after injection. Figure 15D shows whole body imaging of an RFP-labeled *Salmonella* growing in the GFP-labeled PC-3 human prostate tumor four days after injection.

[0031] Figure 16 shows RFP-labeled *Salmonella* targeting and progressively growing in GFP-labeled PC-3 human prostate tumor growing in nude mice demonstrated by histology. RFP-labeled *Salmonella* growing in the GFP-labeled PC-3 human prostate tumor four days after injection (Figure 15D).

[0032] Figures 17A-17B shows the effect of treatment of RFP-labeled *Salmonella* on PC-3 human prostate tumor growing in nude mice demonstrated by histology. Figure 17A is the untreated control. Figure 17B is the treatment after RFP-labeled *Salmonella*.

#### Modes of Carrying Out the Invention

5 [0033] The invention provides model systems for the study of the mechanism of infection. Advantage is taken of visible marker fluorescence proteins to label the infectious agents so that their migration and colonization in tissues can be followed as the infection progresses.

[0034] As used herein, "progression of infection" refers to the general time-dependent manner in which infective agent and infected cells migrate and/or proliferate through an infected  
10 organism. The progress of infection may be a function simply of the location of the infectious agent or infected cells but generally also is a function of the proliferation of the infective agent and infected cells. Thus, both the location and intensity of fluorescence are significant in monitoring progression.

[0035] Since sufficient intensity can be achieved to observe the migration of fluorescent  
15 cells in the intact animal, in addition to determining the migration of the infectious agent by excising organs or tissue, if desired, the progression of metastasis can be observed in the intact subject. Either or both methods may be employed to observe the progress of infection and in evaluating, in model systems, the efficacy of potential protocols and drugs.

[0036] In addition, the present invention takes advantage of delivering therapeutics by  
20 infective agents to tumors and provides an accurate tumor targeting mechanism. It is advantageous in tumor targeting to be able to visualize the infective agent as well as its therapeutic molecule. Some advantages of fluorescence guided injection of tumors are that there is no lower limit to the size of tumor that can be treated, and further, the method is independent of tumor necrosis. In addition, infective agents are not limited to anaerobes nor non-virulent  
25 strains of infective agents. A "therapeutic," "therapeutic molecule" or "therapeutic product" as used herein refers to a gene of interest that is contained in an infective agent, or a product secreted from the infective agent, such as a toxin or other therapeutic protein, or a product that is not secreted but which is used by the infective agent such that a therapeutic effect on tumor is affected. A gene of interest means any gene that has a therapeutic effect on tumor such as a  
30 gene that expresses an anti-tumor agent. Examples of a therapeutic molecule is a gene expressing methioninase or methioninase itself as disclosed in U.S. Pat. No. 6,231,854. Other

examples include p53, BAX, toxins, tumor necrosis factor (TNF), TNF-related apoptosis-inducing ligand, Fas ligand, and antibodies against death receptors.

[0037] The label used in the various aspects of the invention is a fluorescent protein. The native gene encoding the seminal protein in this class, green fluorescent protein (GFP) has been cloned from the bioluminescent jellyfish *Aequorea victoria* (Morin, J., *et al.*, *J. Cell Physiol* (1972) 77:313-318). The availability of the gene has made it possible to use GFP as a marker for gene expression. The original GFP itself is a 283 amino acid protein with a molecular weight of 27 kD. It requires no additional proteins from its native source nor does it require substrates or cofactors available only in its native source in order to fluoresce. (Prasher, D.C., *et al.*, *Gene* (1992) 111:229-233; Yang, F., *et al.*, *Nature Biotechnol* (1996) 14:1252-1256; Cody, C.W., *et al.*, *Biochemistry* (1993) 32:1212-1218.) Mutants of the original GFP gene have been found useful to enhance expression and to modify excitation and fluorescence, so that "GFP" in various colors, including reds and blues has been obtained. GFP-S65T (wherein serine at 65 is replaced with threonine) is particularly useful in the present invention method and has a single excitation peak at 490 nm. (Heim, R., *et al.*, *Nature* (1995) 373:663-664); U.S. Patent No. 5,625,048. Other mutants have also been disclosed by Delagrade, S., *et al.*, *Biotechnology* (1995) 13:151-154; Cormack, B., *et al.*, *Gene* (1996) 173:33-38 and Cramer, A., *et al.*, *Nature Biotechnol* (1996) 14:315-319. Additional mutants are also disclosed in U.S. Patent No. 5,625,048. By suitable modification, the spectrum of light emitted by the GFP can be altered. Thus, although the term "GFP" is often used in the present application, the proteins included within this definition are not necessarily green in appearance. Various forms of GFP exhibit colors other than green and these, too, are included within the definition of "GFP" and are useful in the methods and materials of the invention. In addition, it is noted that green fluorescent proteins falling within the definition of "GFP" herein have been isolated from other organisms, such as the sea pansy, *Renilla reniformis*. Any suitable and convenient form of GFP can be used to modify the infectious agents useful in the invention, both native and mutated forms.

[0038] In order to avoid confusion, the simple term "fluorescent protein" will be used; in general, this is understood to refer to the fluorescent proteins which are produced by various organisms, such as *Renilla* and *Aequorea* as well as modified forms of these native fluorescent proteins which may fluoresce in various visible colors, such as red, yellow, and cobalt, which are exhibited by red fluorescent protein (RFP), yellow fluorescent protein (YFP) or cobalt

fluorescent protein (CFP), respectively. In general, the terms, "fluorescent protein" and "GFP" or "RFP" are used interchangeably.

[0039] Because fluorescent proteins are available in a variety of colors, imaging with respect to more than a single color can be done simultaneously. For example, two different infective agents or three different infective agents each expressing a characteristic fluorescence can be administered to the organism and differential effects of proposed treatments evaluated. In addition, a single infectious organism could be labeled constitutively with a single color and a different color used to produce a fusion with a gene product either intracellular or that is secreted. Thus, the nucleotide sequence encoding a fluorescent protein having a color different from that used to label the organism *per se* can be inserted at a locus to be studied or as a fusion protein in a vector with a protein to be studied. As a further illustration, toxins and other potentially therapeutic proteins will be genetically linked with RFP in order to label and visualize the therapeutic product of GFP-labeled bacteria and visa versa. Two-color imaging will be used to visualize targeting of the bacteria to the tumor as well as their secreted therapeutic product. These tumor-targeting bacteria will be adapted for selective growth in tumors as visualized by their fluorescence. Further, one or more infective agents could each be labeled with a single color, a gene of interest with another color, and the tumor with a third color. For example, fluorescence-expressing tumors in laboratory animals will enable visualization of tumor targeting of fluorescence-labeled infective agents by whole body imaging, as well as the infective agents' therapeutic product.

[0040] As exemplified herein, GFP-and RFP-labeled bacteria were delivered by fluorescence-guided injection in GFP- and RFP-labeled tumors implanted in nude mice and thus the bacteria was targeted to GFP-labeled tumor, thereby inducing tumor necrosis. In particular, the targeting of GFP-and RFP-labeled *E. coli* and *S. typhimurium* to RFP- and- GFP- expressing tumors in mice was visualized by dual-color whole-body imaging. GFP-and RFP-labeled bacteria growing in targeted RFP-and GFP- labeled tumors have been visualized by dual-color whole-body imaging as shown in the Examples herein. Thus, tumor targeting of fluorescent labeled microorganisms has been shown. The method of the invention can also be used, however, to monitor the mis-targeting of the infective agent in order ultimately to select for bacteria that targets tumors.

[0041] Techniques for labeling cells in general using GFP are disclosed in U.S. 5,491,084 (*supra*).

[0042] The methods of the invention utilize infectious agents which have been modified to express the nucleotide sequence encoding a fluorescent protein, preferably of sufficient fluorescence intensity that the fluorescence can be seen in the subject without the necessity of any invasive technique. While whole body imaging is preferred because of the possibility of real-time observation, endoscopic techniques, for example, can also be employed or, if desired, tissues or organs excised for direct or histochemical observation.

[0043] The nucleotide sequence encoding the fluorescent protein may be introduced into the infectious agent by direct modification, such as modification of a viral genome to locate the fluorescent protein encoding sequence in a suitable position under the control sequences endogenous to the virus, or may be introduced into microbial systems using appropriate expression vectors. Infective agents may be bacteria, eukaryotes such as yeast, protozoans such as malaria, or viruses. A multiplicity of expression vectors for particular types of bacterial, protozoan, and eukaryotic microbial systems is well known in the art. A litany of control sequences operable in these systems is by this time well understood. The infectious agent is thus initially modified either to express the fluorescent protein under control of a constitutive promoter as a constant feature of cell growth and reproduction, or may be placed in the microbial or viral genome at particular desired locations, replacing endogenous sequences which may be involved in virulence or otherwise in the progress of infection to study the temporal and spatial parameters characteristic of expression of these endogenous genes. Thus, it is possible to explore the types of factors endogenous to the microbe or virus which contribute to the effectiveness of the infection by suitable choice of positioning. Similarly, a gene expressing a fluorescent protein may be introduced into tumor cells such that laboratory animals contain tumors that can be visualized. Another approach to prepare fluorescent tumors is through photodynamic therapy (PDT) where the tumor absorbs agents that fluoresce such as clinically approved agents, for example, hematoporphorins

[0044] The appropriately modified infectious agent is then administered to the subject in a manner which mimics, if desired, the route of infection believed used by the agent or by an arbitrary route. Administration may be by injection, gavage, oral, by aerosol into the respiratory system, by suppository, by contact with a mucosal surface in general, or by any suitable means known in the art to introduce infectious agents. In tumor targeting where the tumor expresses a fluorescent protein, administration can be made by fluorescent guided injection. Unlike the situation with regard to the study of tumor metastasis using fluorescence, it is not necessary that

the subject be immunocompromised since infection occurs readily in organisms with intact immune systems. However, immunocompromised subjects may also be useful in studying the progress of the condition.

[0045] Although endoscopy can be used as well as excision of individual tissues, it is particularly convenient to visualize the migration of infective agent and infected cells in the intact animal through fluorescent optical tumor imaging (FOTI). This permits real-time observation and monitoring of progression of infection on a continuous basis, in particular, in model systems, in evaluation of potential anti-infective drugs and protocols. Thus, the inhibition of infection observed directly in test animals administered a candidate drug or protocol in comparison to controls which have not been administered the drug or protocol indicates the efficacy of the candidate and its potential as a treatment. In subjects being treated for infection, the availability of FOTI permits those devising treatment protocols to be informed on a continuous basis of the advisability of modifying or not modifying the protocol. In one embodiment, to ascertain the feasibility of fluorescently-labeled bacteria to target tumors, GFP-labeled bacteria were injected into the Lewis lung tumor growing in nude mice. The tumor area became highly fluorescent and readily visualized by blue light excitation in a light box with a CCD camera and a GFP filter.

[0046] Suitable vertebrate subjects for use as models are preferably mammalian subjects, most preferably convenient laboratory animals such as rabbits, rats, mice, and the like. For closer analogy to human subjects, primates could also be used. Any appropriate vertebrate subject can be used, the choice being dictated mainly by convenience and similarity to the system of ultimate interest. Ultimately, the vertebrate subjects can be humans.

[0047] It is expected that tumor-targeting bacteria can be adapted for selective growth in tumors as vectors for tumor-selective gene therapy.

[0048] The following examples are intended to illustrate but not to limit the invention.

#### Preparation A

#### Modification of Infectious Agents

[0049] A variant of the *Renilla mulleri* green fluorescent protein (RMV-GFP) (Zhao, M., Xu, M., Hoffman, R.M., unpublished data) was cloned into the BamHI and NotI sites of the pUC19 derivative pPD16.38 (Clontech, Palo Alto, CA) with GFP expressed from the *lac* promoter. The vector was termed pRMV-GFP. pRMV-GFP was transfected into *E. coli* JM



109 competent cells (Stratagene, San Diego, CA) by standard methods, and transformed cells were selected by ampicillin resistance on agar plates. High expression *E. coli*-GFP clones were selected by fluorescence microscopy.

[0050] *E. coli* has also been labeled with RFP and, in addition, *Salmonella typhimurium* has been labeled with both the GFP and RFP.

### Example 1

#### Infection of Mice by Gavage

[0051] *Nu/nu*/CD-1 mice, 4 weeks old, female, mice were gavaged with 0.5 ml of an *E. coli*-GFP suspension ( $5 \times 10^{10}$ /ml) with a 20 gauge barrel tip feeding needle (Fine Science Tools Inc., Foster City, CA) and latex-free syringe (Becton Dickinson, Franklin Lakes, NJ).

[0052] After gavage, at various time points, imaging of the mice was performed. Imaging was carried out in a light box illuminated by blue light fiber optics (Lighttools Research, Inc., Encinitas, CA). Images were captured using a Hamamatsu C5810 3-chip cooled color CCD camera (Hamamatsu Photonics Systems, Bridgewater, NJ). Images of 1024 x 724 pixels were captured directly on an IBM PC or continuously through video output on a high resolution Sony VCR model SLV-R1000 (Sony Corp., Tokyo, Japan). Images were processed for contrast and brightness and analyzed with the use of Image Pro Plus 3.1 software (Media Cybernetics, Silver Springs, MD).

[0053] *E. coli*-GFP introduced to the mouse GI tract by gavage became visible in the stomach in whole body images almost immediately (Figure 1A). The stomach emptied within 10 minutes post gavage and the *E. coli*-GFP next appeared in the small intestine (Figure 1B-G). The bacterial population in the small intestine appeared to peak at 40 minutes post gavage (Figure 1E) and disappeared by 120 minutes (Figure 1G). After 120 minutes, *E. coli*-GFP appeared in the colon (Figure 1H).

[0054] At appropriate times after gavage, the abdominal cavity was opened and intravital images made of the *E. Coli*-GFP fluorescence. The stomach (Figure 2A), small intestine (Figure 2B), and colon (Figure 2C) were brightly fluorescent with *E. coli*-GFP as seen by intravital imaging. Multiple gavage with *E. coli*-GFP allowed simultaneous inoculation of the stomach, small intestine, and colon, which were imaged by whole-body (Figure 3A) and intravital techniques (Figure 3B). Comparison of whole-body and intravital images of *E. coli*-GFP in the stomach, small intestine, and colon showed a high degree of correspondence.

### Example 2

#### *E. coli*-GFP Direct Colon Infection

[0055] One and one half ml containing  $3 \times 10^{10}$  *E. coli*-GFP per mouse were administered into the colon by enema using a 20 gauge barrel tip feeding needle (Fine Science Tools Inc., Foster City, CA) and latex-free syringe (Becton Dickinson). These mice were also subjected to imaging using the techniques of Example 1. The results are shown in Figure 4.

### Example 3

#### *E. coli*-GFP Peritoneal Infection and Response to Antibiotics

[0056] The mice in each group were given an intraperitoneal (i.p.) injection of  $10^9$ - $10^{10}$  *E. coli*-GFP using a 1 ml 29G1 latex-free syringe (Becton Dickinson). Immediately after injection, the fluorescent bacteria were seen localized around the injection site by external whole-body imaging. (Figure 5A, C). Six hours later, the *E. coli*-GFP were seen to spread throughout the peritoneum (Figure 5B), coinciding with the death of the animal. Intravital imaging of *E. coli*-GFP in the open peritoneal cavity at 6 hours (Figure 6) showed a bacterial distribution similar to that seen by external whole-body imaging.

[0057] Another group of intraperitoneally infected animals were treated with 2 mg Kanamycin in 100  $\mu$ l following inoculation. A control group of infected mice were given an i.p. injection of 100  $\mu$ l of PBS instead of antibiotic. Whole-body imaging of treated mice showed a marked reduction of the bacterial population over the next six hours (Figure 5C, D).

### Example 4

#### Targeting in Brain Cancer Using Whole-Body Imaging

[0058] A PBS solution (10  $\mu$ l) containing  $1 \times 10^8$  GFP-labeled *Salmonella typhimurium* was injected in the RFP-labeled U-87 human glioma in a nude mouse (Figure 7A) using fluorescence guided injection (Figure 7B). GFP-labeled *Salmonella* in the RFP-labeled U-87 human glioma was imaged using techniques similar to Example 1 immediately after injection (Figure 7C). GFP-labeled *Salmonella* growing in the RFP-labeled U-87 human glioma one day after injection was seen showing GFP-labeled *Salmonella* localization around the tumor as well as reduction of tumor size (Figure 7D).

Example 5Targeting in Prostate Tumors Using Whole-Body Imaging

[0059] In a first nude mouse having an RFP-labeled DU-145 human prostate tumor (Figure 8A),  $1 \times 10^8$  GFP-labeled *Salmonella typhimurium* was injected in the RFP-labeled DU-145 human prostate tumor and imaged using whole-body imaging immediately after injection (Figure 8B). GFP-labeled *Salmonella* localization around the tumor (Figure 8B) was seen. One advantage to fluorescent guided injection is that virulent *Salmonella* can be used and tumors of all sizes can be targeted.

[0060] In a second nude mouse having an RFP-labeled DU-145 human prostate tumor (Figure 8C), a solution containing  $2 \times 10^8$  GFP-labeled *Salmonella typhimurium* was injected in the RFP-labeled DU-145 human prostate tumor and imaged using whole-body imaging immediately after injection. GFP-labeled *Salmonella typhimurium* localization in the tumor (Figure 8D) was seen.

Example 6Targeting in Breast Cancer Using Whole-Body Imaging

[0061] A solution containing  $2 \times 10^8$  GFP-labeled *Salmonella typhimurium* was injected in the RFP-labeled MDA MB-435 human breast tumor growing in a nude mouse (Figure 9A) and imaged using techniques similar to Example 1 immediately after injection showing localization around the tumor (Figure 9B) and apparent reduction of tumor size, indicating tumor necrosis.

Example 7Targeting in Prostate Tumor Using Whole-Body Imaging

[0062] A solution containing  $3 \times 10^8$  RFP-labeled *Salmonella typhimurium* was injected in the GFP-labeled PC-3 human prostate tumor growing in a nude mouse (Figure 10A) and imaged using techniques similar to Example 1 immediately after injection (Figure 10B) Growth of RFP-labeled *Salmonella typhimurium* in the GFP-labeled PC-3 human prostate tumor was seen one day after injection (Figure 10C) showing RFP-labeled *Salmonella* growth around the tumor and reduction of tumor size.

Example 8Targeting in Prostate Tumor Using Whole-Body Imaging

[0063] A solution containing  $2 \times 10^8$  RFP-labeled *Salmonella typhimurium* was injected in the GFP-labeled PC-3 human prostate tumor growing in a nude mouse (Figure 11A) and imaged using techniques similar to Example 1 immediately after injection (Figure 11B). RFP-labeled *Salmonella* was detected as growing in the GFP-labeled PC-3 human prostate tumor one day after injection (Figure 11C) and continuing to grow in the tumor four days after injection (Figure 11D) while reduction of tumor size is shown.

Example 9Targeting in Prostate Tumor Using Whole-Body Imaging

[0064] A solution containing  $2 \times 10^8$  RFP-labeled *Salmonella typhimurium* was injected in the GFP-labeled PC-3 human prostate tumor growing in a nude mouse (Figure 12A) and imaged using techniques similar to Example 1 immediately after injection (Figure 12B). RFP-labeled *Salmonella* is seen growing in the GFP-labeled PC-3 human prostate tumor one day after injection (Figure 12C) and four days after injection (Figure 12D) showing visible reduction in tumor size.

[0065] Histological studies were performed on the RFP-labeled *Salmonella* growing in the GFP-labeled PC-3 human prostate tumor four days after injection (Figure 12D) by fixing the tumor tissue with 10% buffered formaline and processed for paraffin section and HE staining by standard method. The RFP-labeled *Salmonella* (the small blue dots as pointed by the white arrows in Figure 13, Magnification 400X) were progressively growing in the PC-3 tumor tissue and targeting the tumor cells.

[0066] Histological studies (HE staining, Magnification 200X) also compared an untreated control showing a well-maintained PC-3 human prostate tumor structure growing in a nude mouse (Figure 14A) with PC-3 human prostate tumor growing in a nude mouse treated with RFP-labeled *Salmonella* four days after injection (Figure 14B). The majority of tumor tissue has been destroyed, and the extensive necrosis (arrows in Figure 14B) in the tumor is shown.

Claims

1. A method to monitor the progression of infection in a vertebrate subject which method comprises

5 observing the presence, absence or intensity of fluorescence at various locations in said subject as a function of time, wherein said vertebrate subject has been treated with an infective agent that expresses a fluorescent protein.

2. The method of claim 1, wherein said observing is by endoscopy or fluorescent optical tumor imaging in the intact subject.

10 3. The method of claim 1, wherein the fluorescent protein has green fluorescence or red fluorescence.

4. The method of claim 1, wherein the infective agent is a bacterium, protozoan or virus.

5. The method of claim 1, wherein the infective agent is a eukaryotic single-celled organism.

15 6. The method of claim 1, wherein the subject is a mammal.

7. The method of claim 6, wherein the subject is a mouse, rat or rabbit.

8. The method of claim 1, wherein the subject is immunocompromised.

9. A method to evaluate a candidate protocol or drug for the inhibition of infection which method comprises

20 administering said protocol or drug to a vertebrate subject which has been treated with an infective agent that expresses a fluorescent protein and monitoring the progression of infection over time by observing the presence, absence or intensity of the fluorescence at various locations in said treated subject;

25 monitoring the progression of infection over time in a control subject which has been similarly treated with an infective agent that expresses a fluorescent protein; and

comparing the progression of infection in said treated subject with the progression of infection in said control subject;

whereby a diminution of the progression of infection in said treated subject as compared to said control subject identifies the protocol or drug as effective in inhibiting infection.

5           10.    The method of claim 9, wherein said monitoring is by endoscopy or fluorescent optical tumor imagining in the intact subject.

          11.    The method of claim 9, wherein the infective agent is a bacterium, protozoan or virus.

10           12.    The method of claim 9, wherein the infective agent is a eukaryotic single-celled organism.

          13.    The method of claim 9, wherein the subject is a mammal.

          14.    The method of claim 13, wherein the subject is a mouse, rat or rabbit.

          15.    The method of claim 9, wherein the subject is immunocompromised.

15           16.    A method to identify genes associated with infection, which method comprises observing the presence, absence or intensity of fluorescence in said subject as a function of time and location in said subject, wherein said subject has been infected with an infective agent with an altered genome, wherein the alteration in the genome comprises replacement of a nucleotide sequence whose function is to be determined with a nucleotide sequence encoding a fluorescent protein.

20           17.    The method of claim 16, wherein the infective agent is a bacterium, protozoan or virus.

          18.    The method of claim 16, wherein the infective agent is a eukaryotic single-celled organism.

          19.    The method of claim 16, wherein the subject is a mammal.

20. The method of claim 19, wherein the subject is a mouse, rat or rabbit.

21. The method of claim 16, wherein the subject is immunocompromised.

22. The method of claim 16, wherein said observing is by endoscopy or fluorescent optical tumor imaging in the intact subject.

5           23. A method of targeting tumors using a therapeutic infective agent in a vertebrate subject comprising  
              administering an infective agent that expresses a fluorescent protein to said vertebrate subject containing a tumor; and  
              observing the presence, absence or intensity of fluorescence in said subject as a function  
10       of time.

24. The method of claim 23, wherein the therapeutic infective agent delivers a therapeutic product to the tumor.

25. The method of claim 23, wherein the tumor exhibits fluorescence of a color other than the color of the infective agent.

15           26. The method of claim 24, wherein the therapeutic product exhibits fluorescence of a color different than the color of the infective agent and the tumor.

27. The method of claim 23, wherein the infective agent is a bacterium, a protozoan or a virus.

20           28. The method of claim 23, wherein the infective agent is a eukaryotic single-celled organism.

29. The method of claim 23, wherein the subject is a mammal.

30. The method of claim 29, wherein the subject is a mouse, rat or rabbit.

31. The method of claim 22, wherein the subject is immunocompromised.

32. The method of claim 22, wherein said observing is by endoscopy or by fluorescent optical tumor imaging in the intact subject.

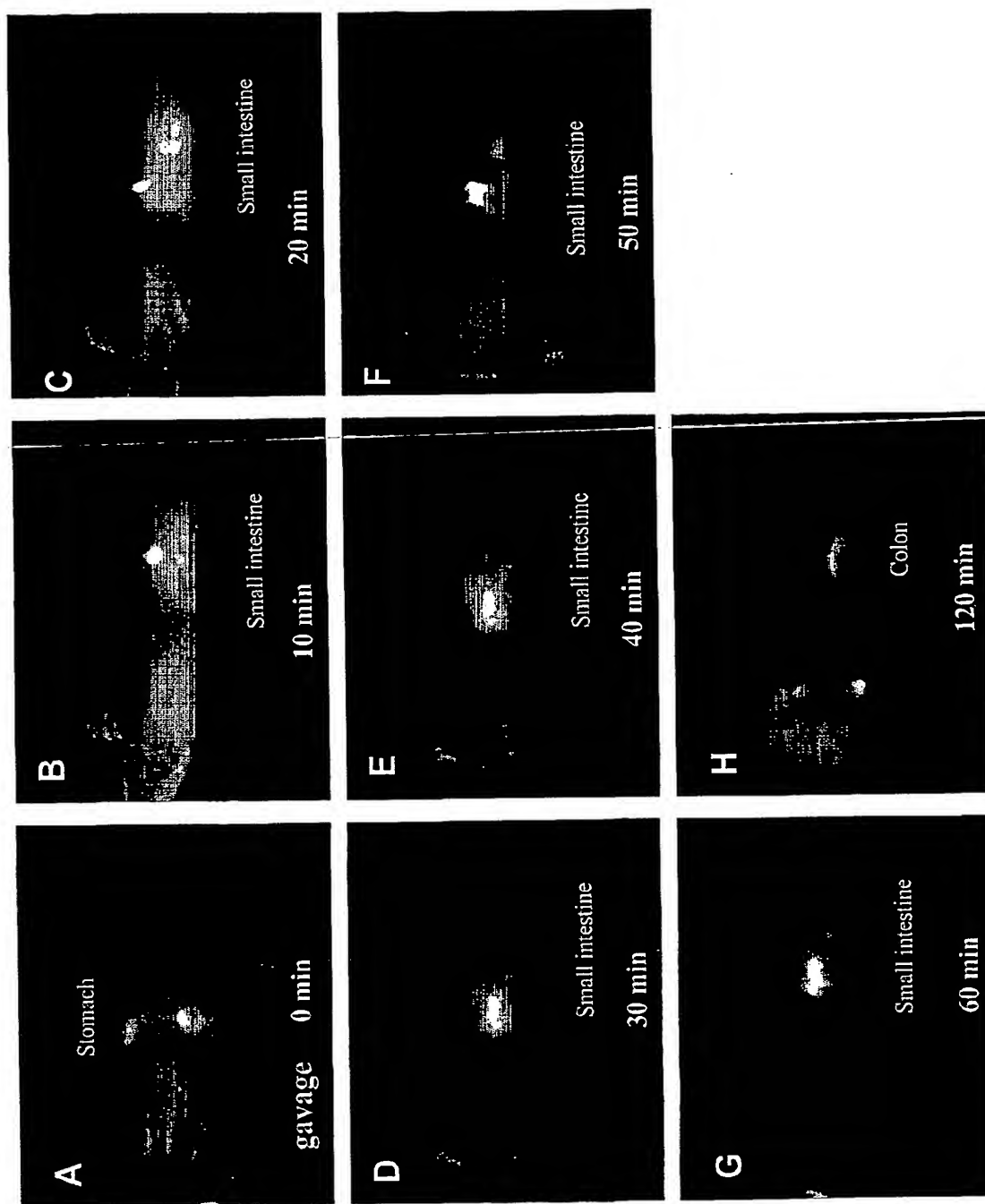
33. The method of claim 23, wherein tumor necrosis is induced.

34. A tumor targeting infective agent comprising an infective agent that expresses a fluorescent protein, where the infective agent is capable of preferentially targeting the tumor in an intact, living mammal in comparison to normal cells.

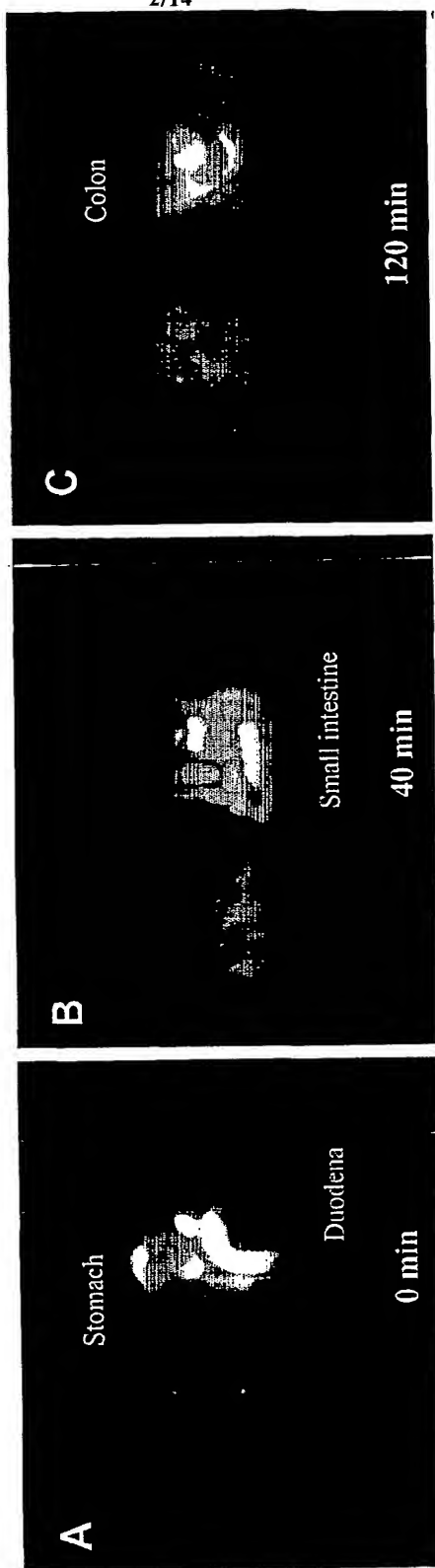
35. A tumor targeting infective agent as in claim 34, wherein the infective agent contains or secretes a therapeutic molecule or contains a gene of interest.



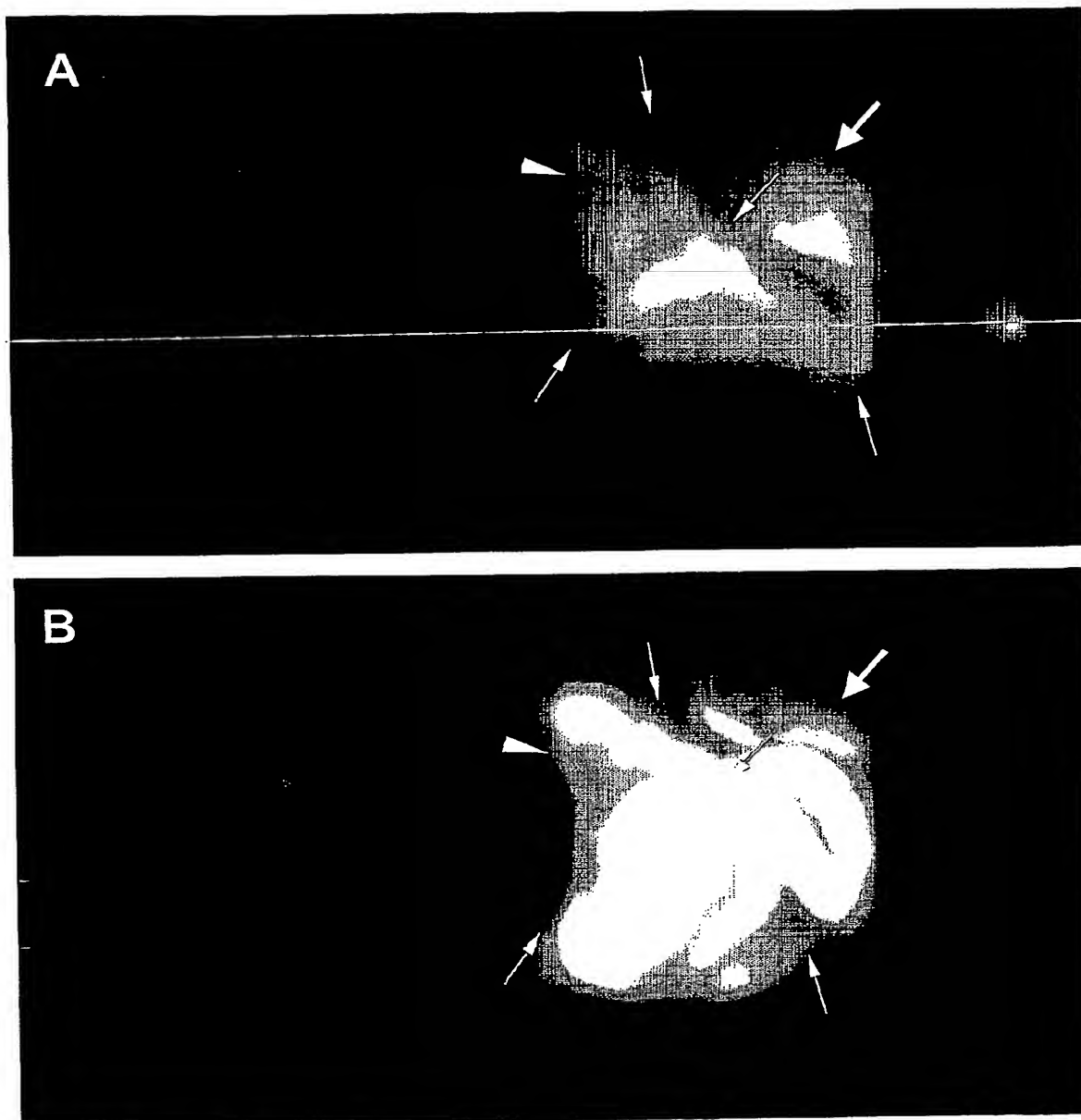
**Figure 1**



**Figure 2**



**Figure 3**



**Figure 4**

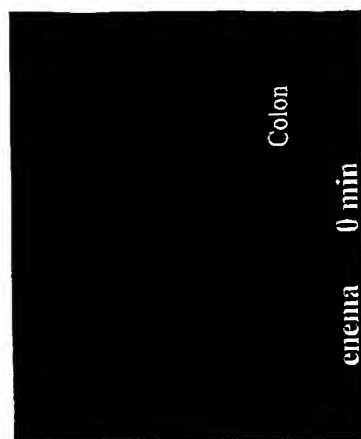
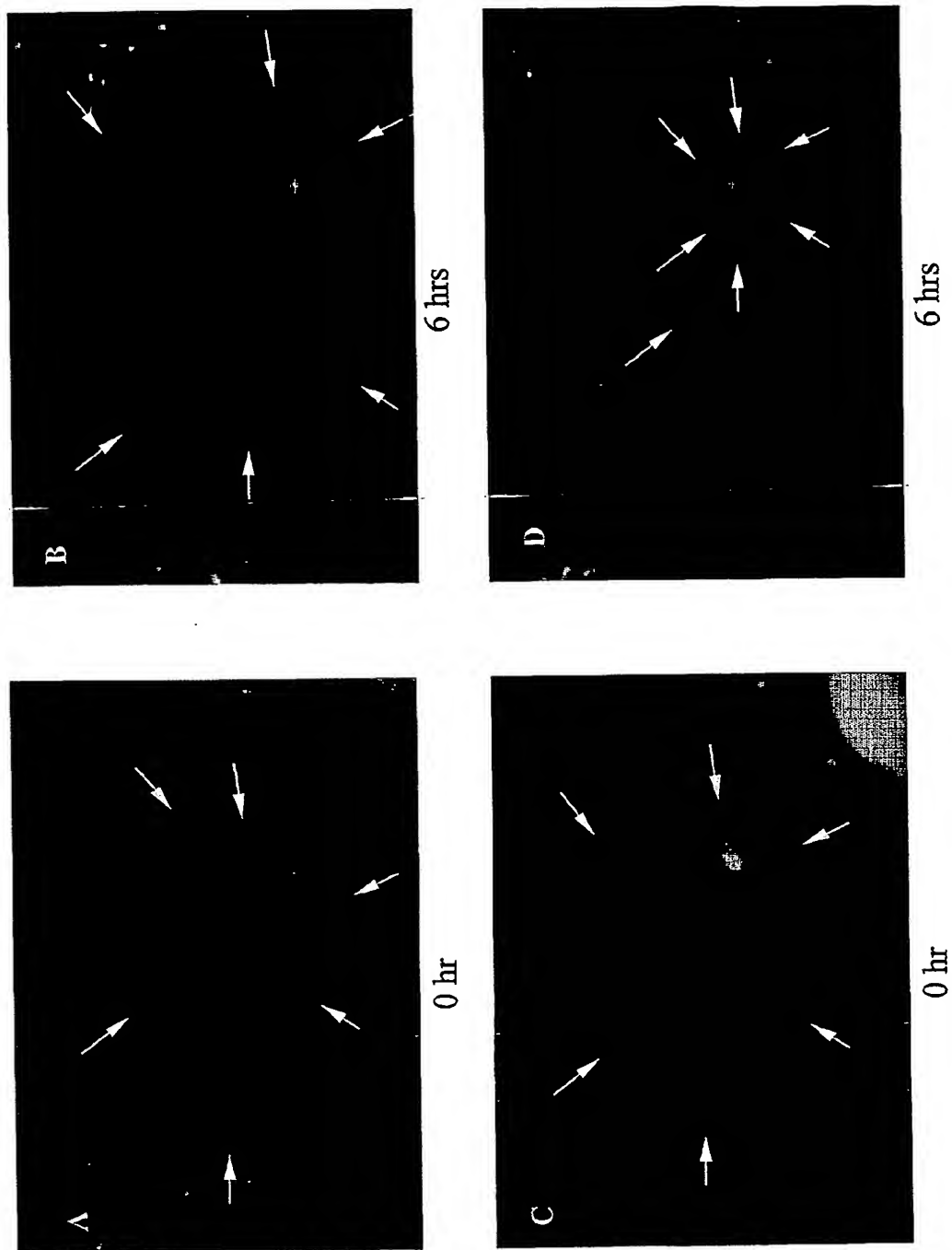


Figure 5



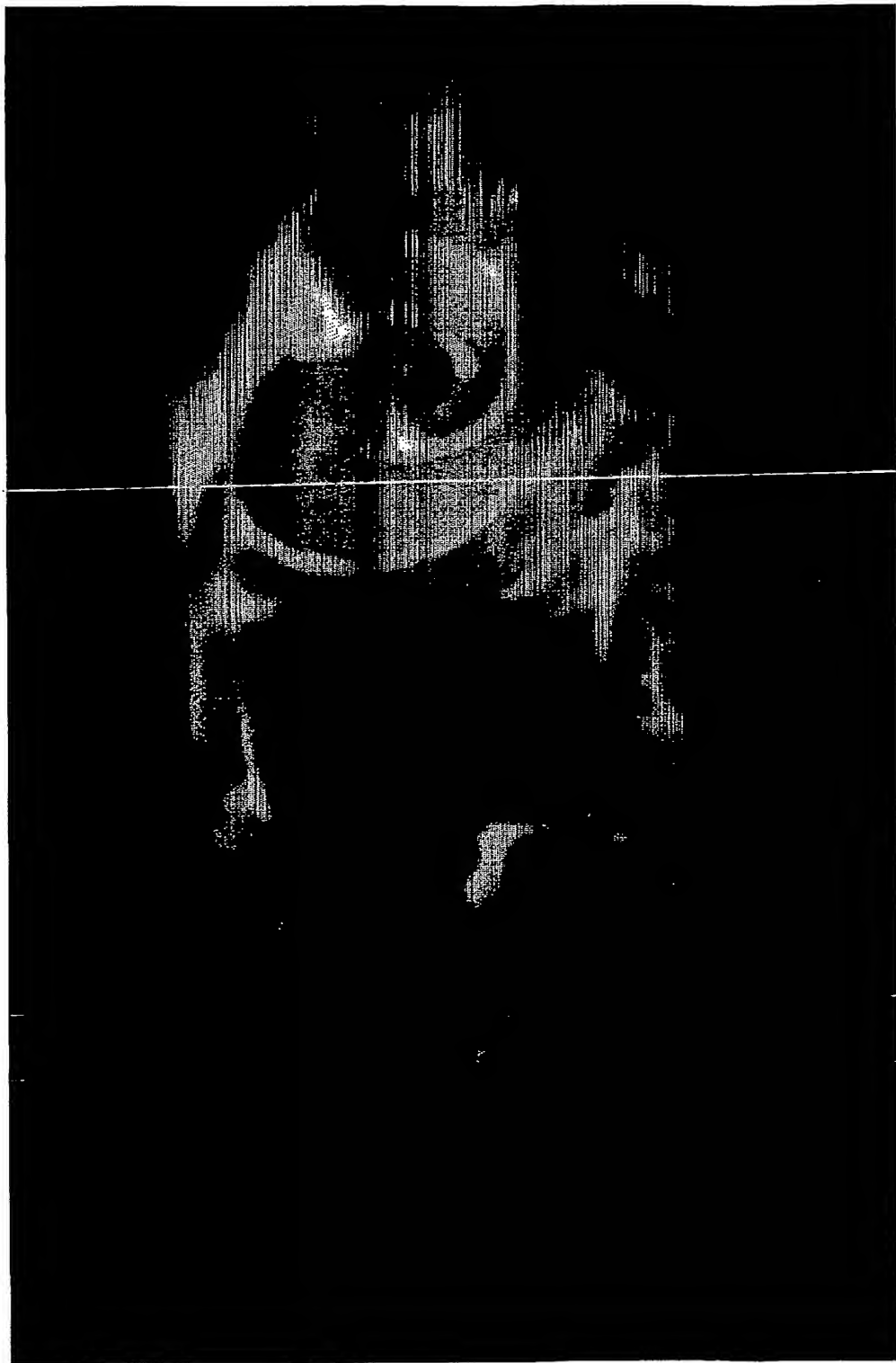
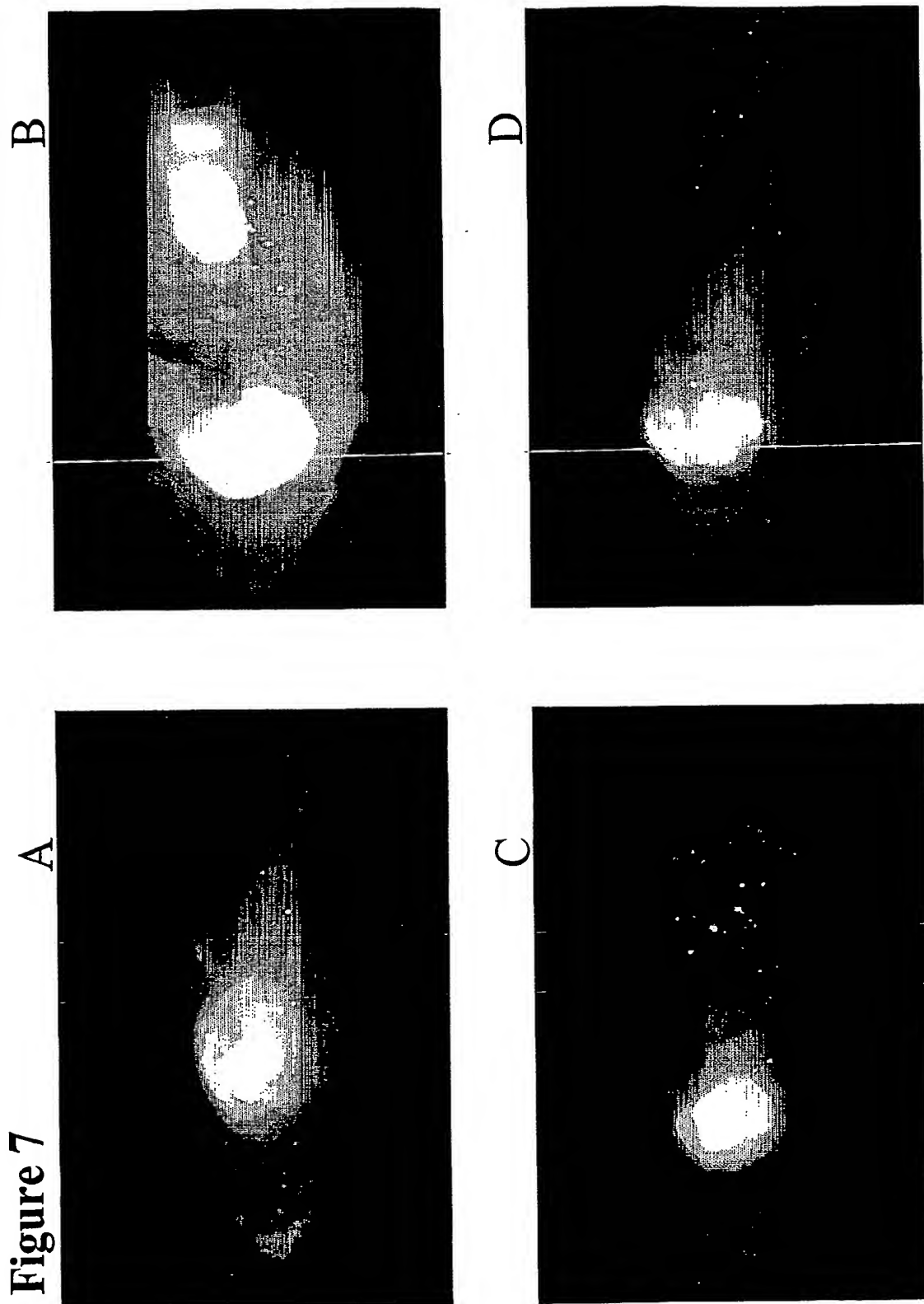
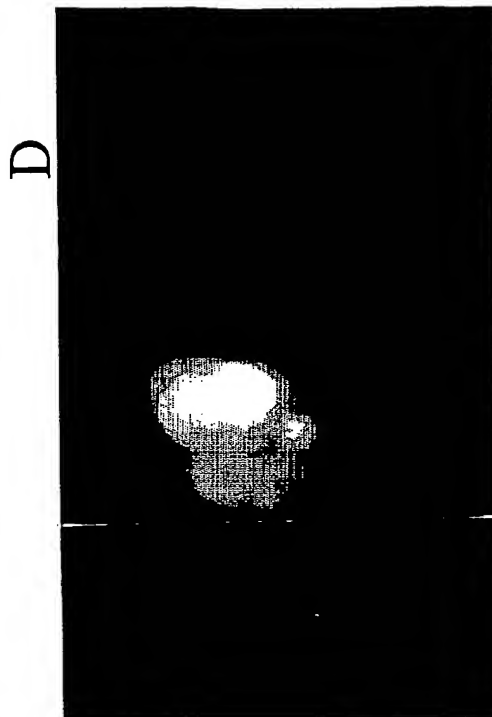
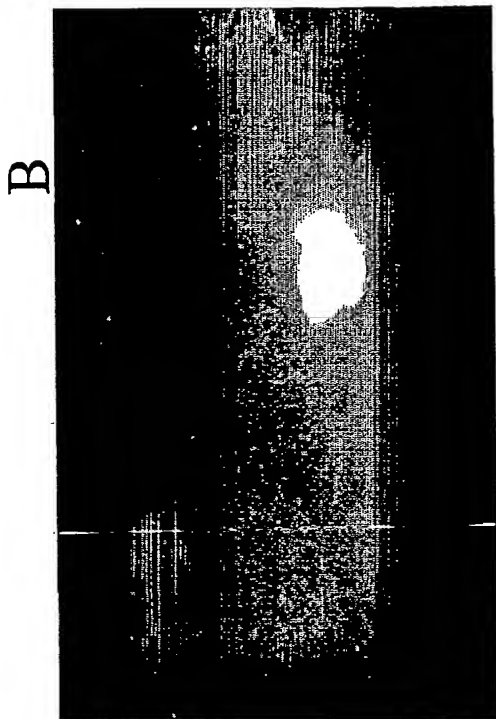
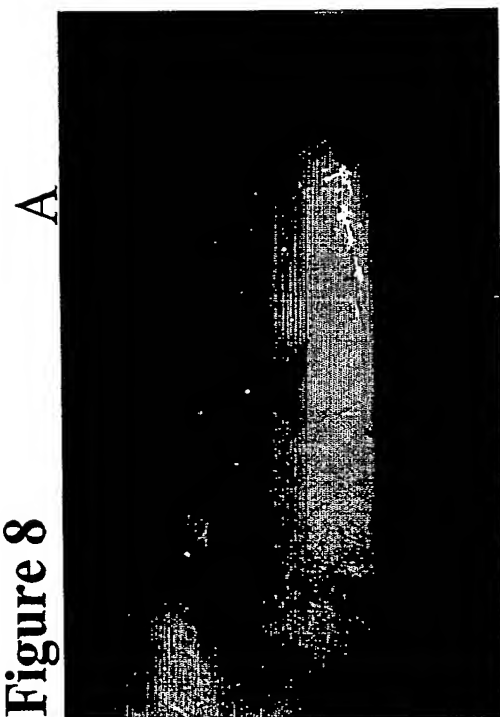


Figure 6



**Figure 8**





9/14

B



A

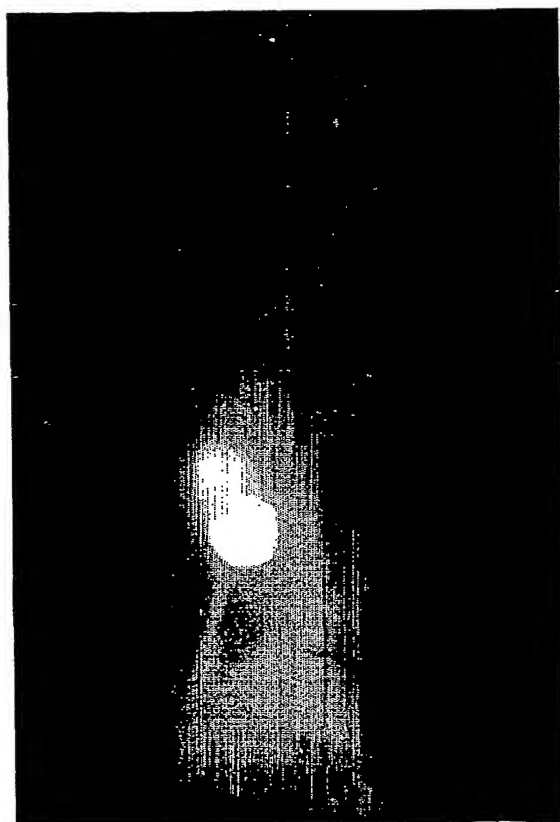
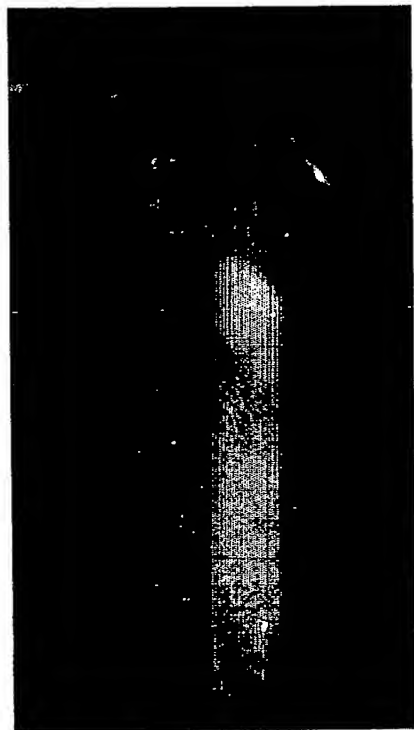
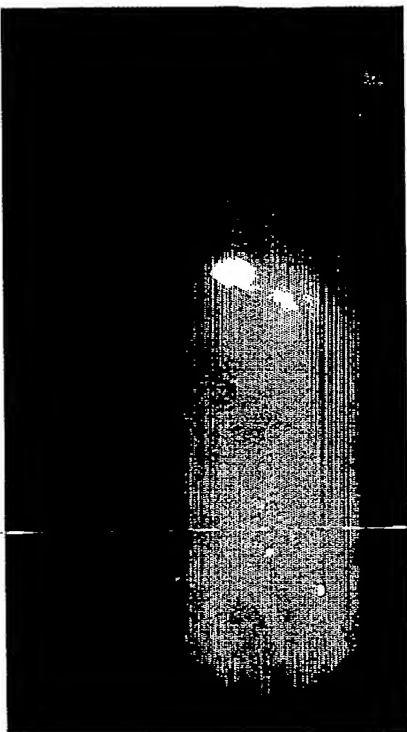


Figure 9

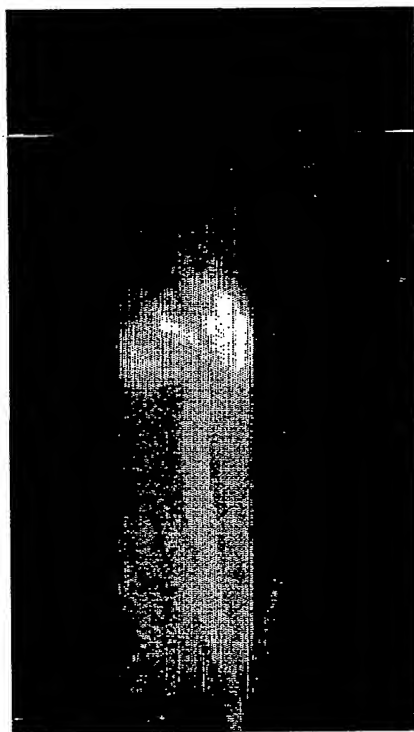
**Figure 10**



A



B



C

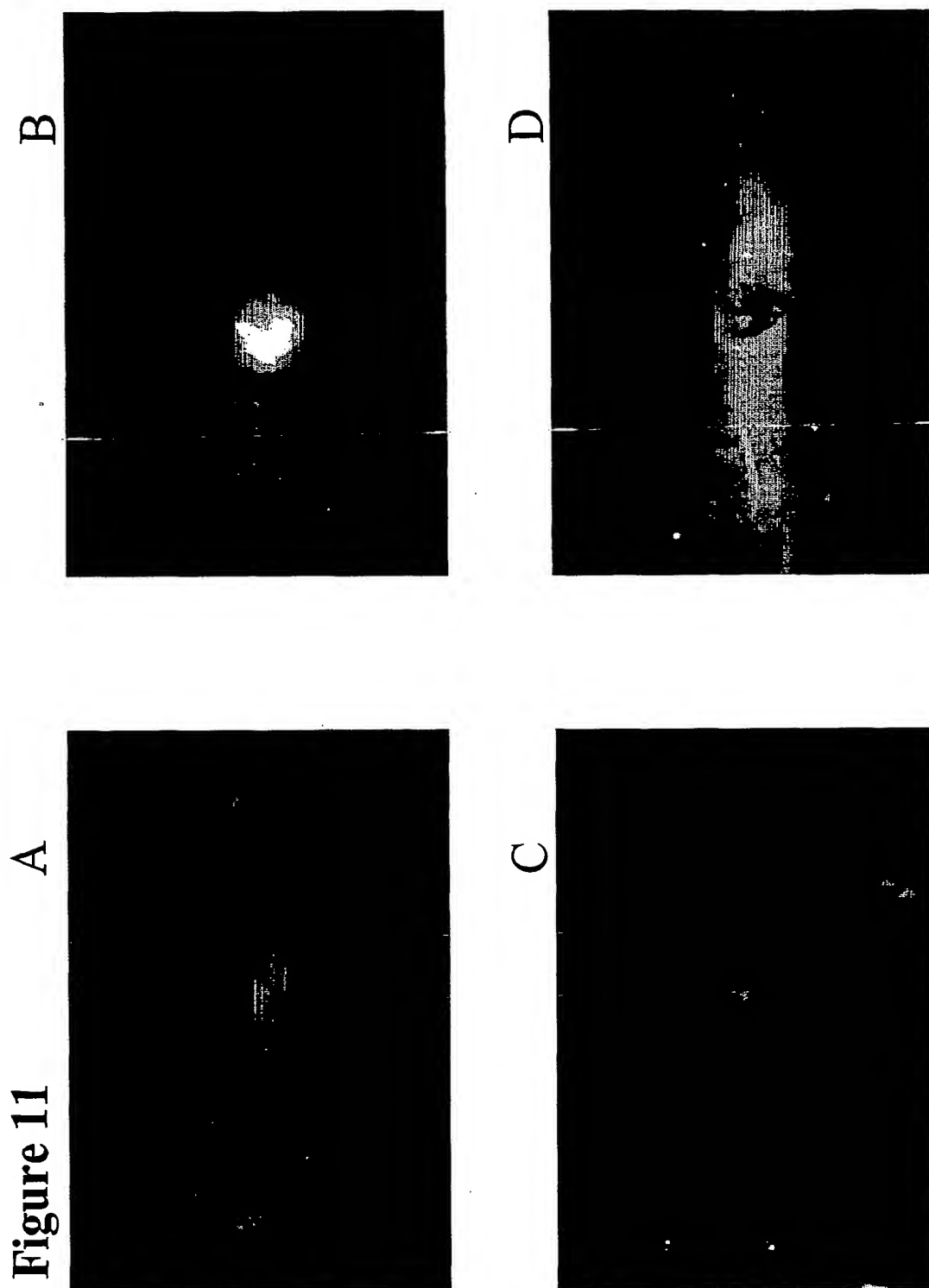
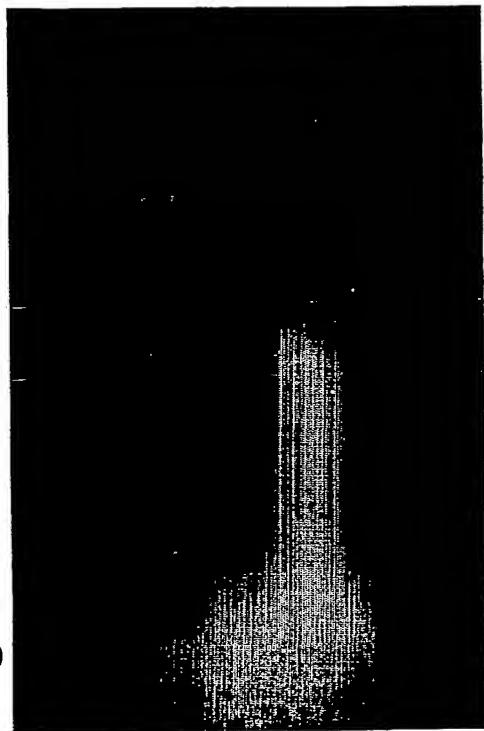
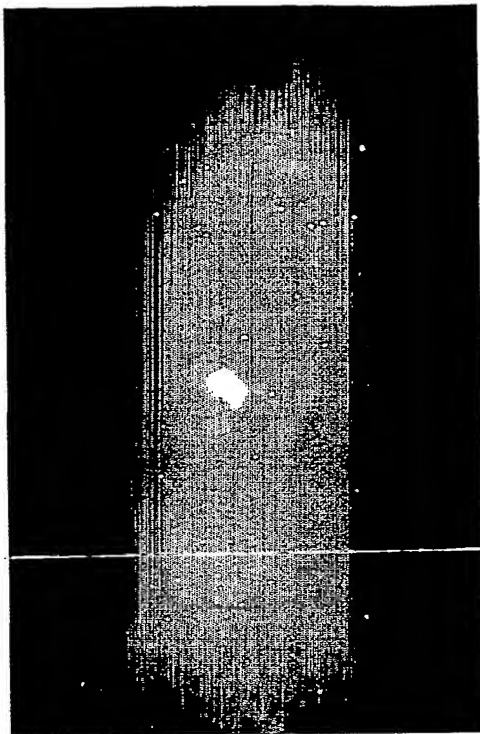


Figure 12

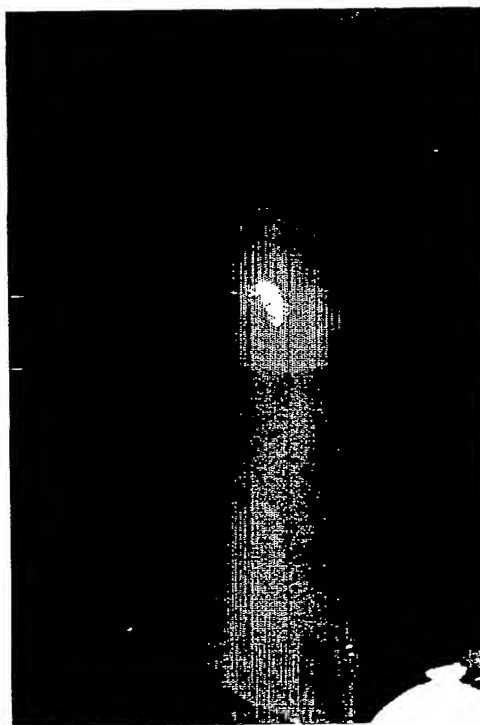
A



B



C



D

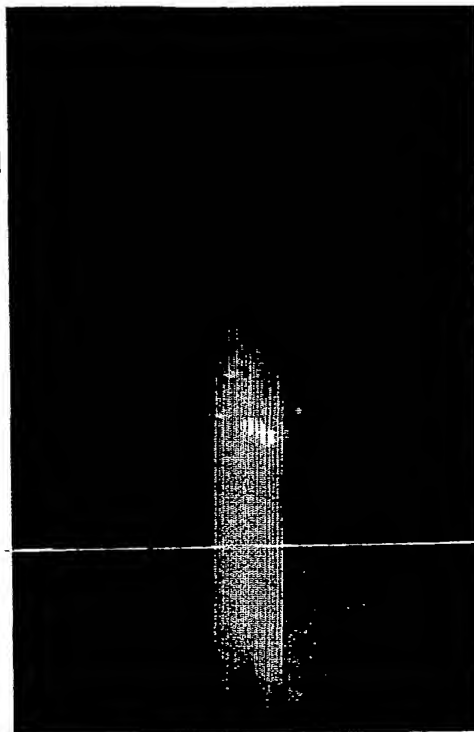
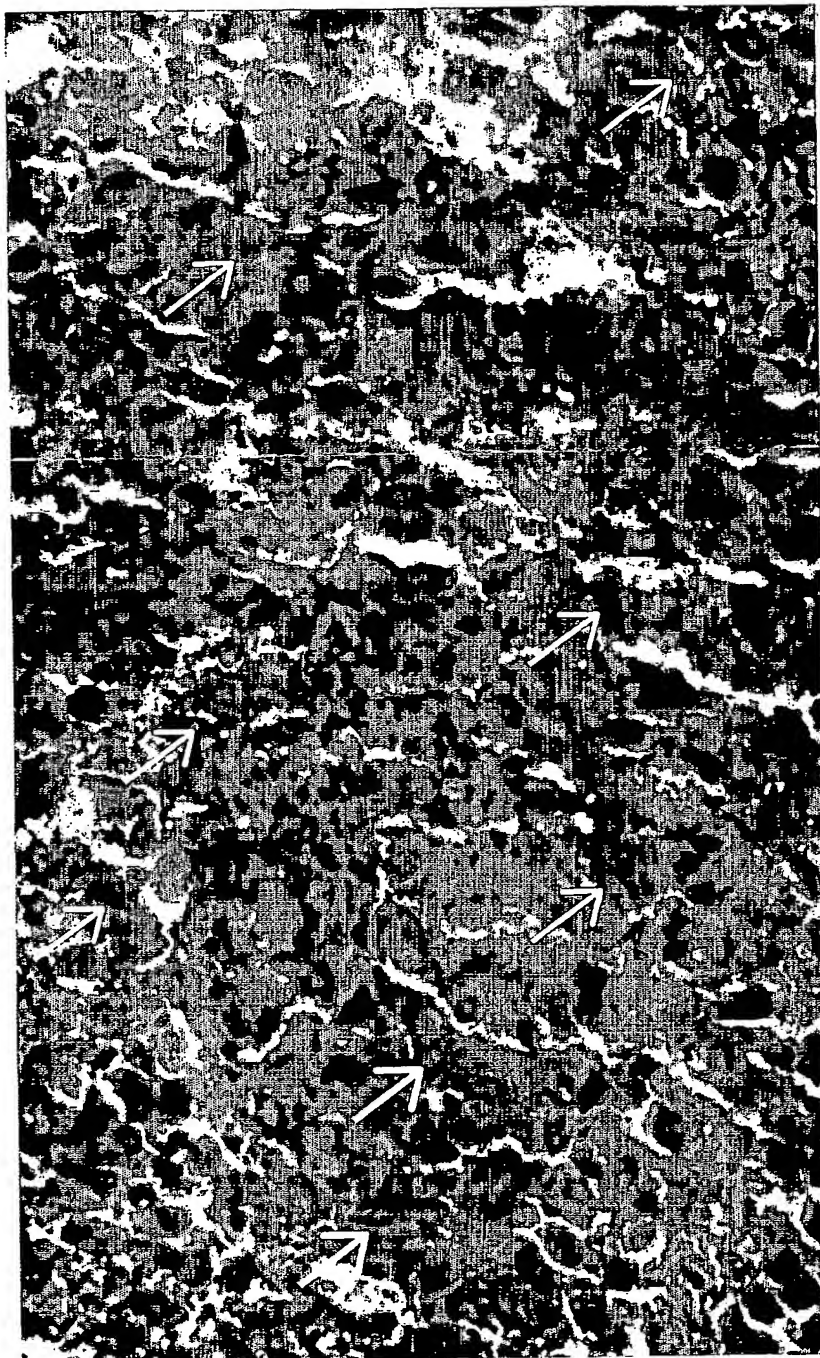
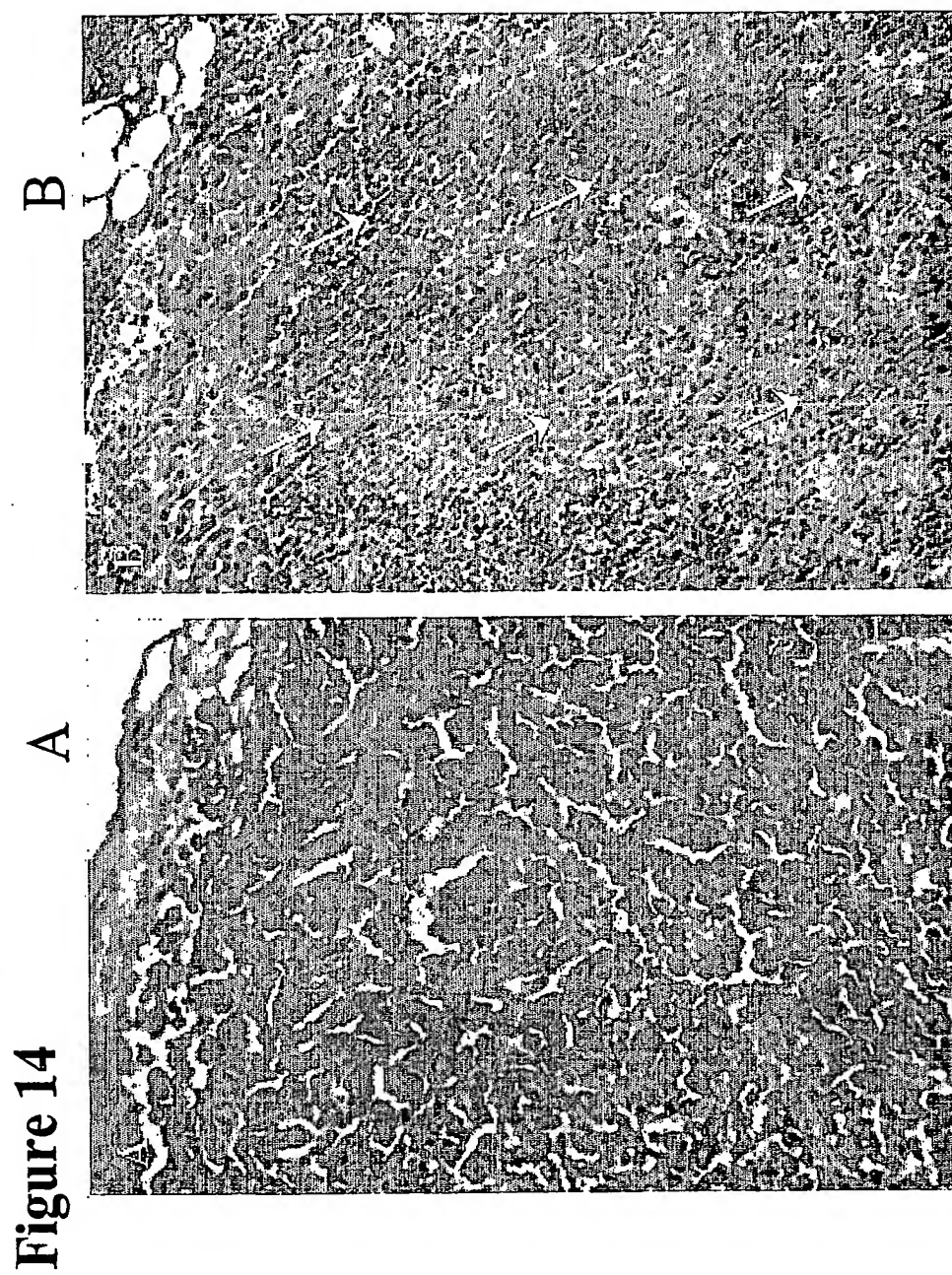


Figure 13





## INTERNATIONAL SEARCH REPORT

In national Application No

PCT/US 02/21812

**A. CLASSIFICATION OF SUBJECT MATTER**  
IPC 7 A61K49/00 C12N1/21

According to International Patent Classification (IPC) or to both national classification and IPC

**B. FIELDS SEARCHED**

Minimum documentation searched (classification system followed by classification symbols)

IPC 7 A61K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the International search (name of data base and, where practical, search terms used)

EPO-Internal, BIOSIS

**C. DOCUMENTS CONSIDERED TO BE RELEVANT**

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No. -
X	MENG YANG ET AL: "WHOLE-BODY OPTICAL IMAGING OF GREEN FLUORESCENT PROTEIN-EXPRESSING TUMORS AND METASTASES" PROCEEDINGS OF THE NATIONAL ACADEMY OF SCIENCES OF USA, NATIONAL ACADEMY OF SCIENCE. WASHINGTON, US, vol. 97, no. 3, 1 February 2000 (2000-02-01), pages 1206-1211, XP000904536 ISSN: 0027-8424 cited in the application abstract  -/-	1-22, 34, 35

☒ Further documents are listed in the continuation of box C.☒ Patent family members are listed in annex.

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- \*A\* document defining the general state of the art which is not considered to be of particular relevance
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Date of the actual completion of the international search

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## INTERNATIONAL SEARCH REPORT

In International Application No  
PCT/US 02/21812

## C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	<p>YANG MENG ET AL: "Visualizing gene expression by whole-body fluorescence imaging"</p> <p>PROCEEDINGS OF THE NATIONAL ACADEMY OF SCIENCES OF USA, NATIONAL ACADEMY OF SCIENCE. WASHINGTON, US, vol. 97, no. 22, 24 October 2000 (2000-10-24), pages 12278-12282, XP002210069 ISSN: 0027-8424 abstract</p>	16,17, 19-22, 34,35
X	<p>WO 01 25399 A (SLOAN KETTERING INST CANCER ;VION PHARMACEUTICALS INC (US)) 12 April 2001 (2001-04-12) abstract page 17, line 3 -page 19, line 7 page 34, line 20 -page 45, line 31; example 6.9</p>	1-35
X	<p>WO 01 05229 A (BRIGHAM &amp; WOMENS HOSPITAL ;CHIOCCA E ANTONIO (US); GEN HOSPITAL CO) 25 January 2001 (2001-01-25) page 8, last paragraph -page 12, line 10; claims; example 1</p>	23-27, 29,31, 33-35
X	<p>US 6 251 384 B1 (CHISHIMA TAKASHI ET AL) 26 June 2001 (2001-06-26) cited in the application column 3, line 1 -line 47; claims; example 8</p>	1-22,34, 35
X	<p>WO 01 18195 A (XENOGEN CORP) 15 March 2001 (2001-03-15) page 28, line 29 -page 29, line 33; examples 6,7</p>	1-22
P,X	<p>ZHAO MING ET AL: "Spatial-temporal imaging of bacterial infection and antibiotic response in intact animals."</p> <p>PROCEEDINGS OF THE NATIONAL ACADEMY OF SCIENCES OF THE UNITED STATES, vol. 98, no. 17, 14 August 2001 (2001-08-14), pages 9814-9818, XP002218906 August 14, 2001 ISSN: 0027-8424 the whole document</p>	1-22,34, 35
P,X	<p>WO 01 71009 A (ANTICANCER INC) 27 September 2001 (2001-09-27) claims; example 1</p>	1-35



# INTERNATIONAL SEARCH REPORT

International application No.  
PCT/US 02/21812

## Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This International Search Report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☒ Claims Nos.:  
because they relate to subject matter not required to be searched by this Authority, namely:  
Although claims 23 to 33 are directed to a method of treatment of the human/animal body, the search has been carried out and based on the alleged effects of the compound/composition.
2. ☐ Claims Nos.:  
because they relate to parts of the International Application that do not comply with the prescribed requirements to such an extent that no meaningful International Search can be carried out, specifically:
3. ☐ Claims Nos.:  
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

## Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

1. ☐ As all required additional search fees were timely paid by the applicant, this International Search Report covers all searchable claims.
2. ☐ As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.
3. ☐ As only some of the required additional search fees were timely paid by the applicant, this International Search Report covers only those claims for which fees were paid, specifically claims Nos.:
4. ☐ No required additional search fees were timely paid by the applicant. Consequently, this International Search Report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

- ☐ The additional search fees were accompanied by the applicant's protest.
- ☐ No protest accompanied the payment of additional search fees.

# INTERNATIONAL SEARCH REPORT

Information on patent family members

International Application No

PCT/US 02/21812

Patent document cited in search report		Publication date	Patent family member(s)	Publication date
WO 0125399	A	12-04-2001	AU 7993600 A WO 0125399 A2	10-05-2001 12-04-2001
WO 0105229	A	25-01-2001	WO 0105229 A1	25-01-2001
US 6251384	B1	26-06-2001	US 6235968 B1 US 6235967 B1 US 6232523 B1 AU 2406900 A EP 1156833 A1 WO 0040274 A1 AU 749338 B2 AU 7164898 A CN 1264429 T EP 0979298 A1 JP 2001517090 T WO 9849336 A1	22-05-2001 22-05-2001 15-05-2001 24-07-2000 28-11-2001 13-07-2000 27-06-2002 24-11-1998 23-08-2000 16-02-2000 02-10-2001 05-11-1998
WO 0118195	A	15-03-2001	AU 7126600 A EP 1212429 A2 WO 0118195 A2	10-04-2001 12-06-2002 15-03-2001
WO 0171009	A	27-09-2001	AU 4929701 A WO 0171009 A2 US 2002013954 A1	03-10-2001 27-09-2001 31-01-2002

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see PCT Gazette No. 10/2003 of 6 March 2003, Section II

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ance Notes on Codes and Abbreviations" appearing at the begin-  
ning of each regular issue of the PCT Gazette.*

(54) Title: IMAGING INFECTION USING FLUORESCENT PROTEIN AS A MARKER

(57) Abstract: A method to follow the progress of infection in vertebrate subjects utilizes infective agents which have been modified to express a fluorescent protein. The method can also monitor expression of genes associated with infective agents during the course of infection. The method may further include targeting tumors with the modified infective agents.

WO 03/006069 A1

## IMAGING INFECTION USING FLUORESCENT PROTEIN AS A MARKER

### Technical Field

5       [0001] The invention relates to the study of microbial and viral infection. Specifically, it concerns systems for studying progress of, and control of, infection in vertebrates and methods for evaluating candidate drugs and targeting tumors.

### Background Art

10       [0002] The use of green fluorescent protein to visualize cancer progression and metastasis is by now well established. See, for example, Hoffman, R.M., *Methods in Enzymology* (1999) 302:20-31 (P. Michael Conn, ed., Academic Press, San Diego). The use of whole body imaging to chart real time progression and to assess the efficacy of proposed protocols for treating tumors is disclosed in U.S. patent 6,251,384, the contents of which are incorporated herein by reference.

15       [0003] The advantages of green fluorescent protein have been noted in that it does not require any substrates or cofactors and its expression in living cells does not apparently cause any biological damage. In addition, the fluorescence emitted makes this a particularly sensitive technique. Indeed, the whole body images obtainable using simple equipment, *e.g.*, 490 nm excitation from a xenon or mercury lamp along with image capture by a CCD color video camera permit real-time investigations of tumor growth and metastasis. See, for example, Yang, M., *et al.*, *Proc. Natl. Acad. Sci. USA* (2000) 97:1206-1211.

20       [0004] The present invention extends the techniques developed in imaging tumor growth and metastasis to the study of infection. Microbial and viral infection can be monitored by labeling the infectious agent with a bright fluorescent protein and the progress of infection monitored. In addition, protocols useful in treating microbial or viral infection can be evaluated by taking advantage of this technique. The materials and methods for obtaining suitable  
25       expression of fluorescent proteins are readily available. For example, Cheng, L., *et al.*, *Gene Therapy* (1997) 4:1013-1022, describe the modification of hematopoietic stem cells with green fluorescent protein (GFP) encoding sequences under control of a retroviral promoter. Although the authors state that human stem cells are transfected with this system only with difficulty, by

using an enhanced form of the GFP, satisfactory brightness could be achieved. Grignani, F., *et al.*, *Cancer Res* (1998) 58:14-19, report the use of a hybrid EBV/retroviral vector expressing GFP to effect high-efficiency gene transfer into human hematopoietic progenitor cells.

[0005] Vectors containing various modified forms of GFP to provide various colors are marketed by Clontech. The Clontech vectors intended for mammalian cell expression place the GFP under control of the cytomegalovirus (CMV) promoter; such expression systems can also be used to label viral infectious agents.

[0006] Attempts have been made to visualize bacteria in mammalian subjects using luciferase as a marker, but because of the low luminosity of this system, whole body imaging is not practical. See, for example, Contag, P.R., *et al.*, *Nat. Med.* (1998) 4:245-247.

[0007] GFP expressing bacteria have been previously employed in a number of studies that were not in intact, living animals (Wu, H., *et al.*, *Microbiol.* (2000) 146:2481-2493; Ling, S.H.M., *et al.*, *Microbiol.* (2000) 146:7-19; Badger, J.L., *et al.*, *Mol. Microbiol.* (2000) 36(1):174-182; Kohler, R., *et al.*, *Mol. Gen. Genet.* (2000) 262:1060-1069; Valdivia, R.H., *et al.*, *Gene* (1996) 173:47-52; Valdivia, R.H., *et al.*, *Science* (1997) 277:2007-2011; Scott, K.P., *et al.*, *FEMS Microbiol. Ltrs.* (2000) 182:23-27; Prachaiyo, P., *et al.*, *J. Food Protect.* (2000) 63:427-433; Geoffroy, M-C., *Applied & Env. Microbiol.* (2000) 66:383-391). An example of such studies was the visualization of the *in vitro* infection of muscle tissue by the pathogenic *E. coli* O157H GFP (Prachaiyo, P., *et al.*, *supra*). Another approach examined the mouse gastrointestinal tract after gavage infection by removal and fixation of the gastrointestinal tissue (Geoffroy, M-C., *supra*). Fish infected with GFP transduced *Edwardsiella tarda* were imaged for infection after removal of their organs (Ling, S.H.M., *et al.*, *supra*). Genes associated with virulence and other infectious processes were evaluated by linkage to GFP expression (Ling, S.H.M., *et al.*, *supra*; Badger, J.L., *et al.*, *supra*; Kohler, R., *et al.*, *supra*; Valdivia, R.H., *et al.*, *supra* (1996).

[0008] The present invention also extends to targeting tumors to deliver therapeutics thereto via infective agents such as microorganisms using fluorescence. Attempts have been made to deliver the anaerobic bacteria *Clostridia novyi* to necrotic regions in tumors (Dang, L.H., *et al.*, *Proc. Natl. Acad. Sci. USA* (2001) 98:15155-15160). In addition, the necrotic regions of tumors have been targeted using *Bifidobacterium longum* (Yazawa, K., *et al.*, *Cancer Gene Therapy* (2000) 7(2):269-274 and Yazawa, K., *et al.*, *Breast Cancer Res. & Treatment* (2001)

66:165-170). These approaches depend on anaerobes, are targeted at necrotic tissue only and/or may be used only for tumors of a large size. Further, tumors have been targeted using *Salmonella* that is devoid of its toxin (Low, K.B., *et al.*, *Nature Biotech.* (1999) 17:37-41). Additional studies have reported the tumor targeting capability of *Salmonella* in human patients with metastatic melanoma and renal cell carcinoma (Toso, J.F., *et al.*, *J. Clin. Oncol.* (2002) 20(1):142-152). These approaches do not provide a way to visualize the bacteria in living animals.

[0009] Bacteria and other microorganisms offer many features to deliver therapeutics to tumors. For example they are readily transformed to produce both human and specialized bacterial proteins. The bacterial proteins, however, include a wide variety and potency of toxins. In order to take advantage of such powerful molecules, it would be useful to have an accurate tumor-targeting mechanism for therapeutic-delivering bacteria as shown by the present invention.

#### Disclosure of the Invention

[0010] The invention provides models which permit the intimate study of formation of microbial or viral infection in a realistic and real-time setting. By using fluorescent proteins such as green fluorescent protein (GFP) as a stable and readily visualized marker, the progression of infection can be modeled and the mechanism elucidated. The invention is also directed, in part, to tumor targeting which depends on the ability to visualize the bacteria or microorganism as well as its therapeutic molecule.

[0011] Thus, in one aspect, the invention is directed to a method to monitor the course of infection in a model vertebrate system by monitoring the spatial and temporal progression of fluorescence in said vertebrate subject wherein said subject has been subjected to infection by a microbe or virus which microbe or virus expresses a fluorescent protein.

[0012] In another aspect, the invention is directed to a method to evaluate a candidate protocol or drug for inhibition of infection in a subject which method comprises administering the protocol or drug to a vertebrate subject which has been infected with a microbe or virus that expresses a fluorescent protein and monitoring the temporal and spatial progress of infection by observing the presence, absence or intensity of fluorescence at various locations at various times in the infected subject. In this method, in addition, the presence, absence or intensity of

fluorescence at various locations in a control subject at various times is also monitored for comparison with the subject that has been treated with the protocol or drug. The progress of infection over time and space is compared in the treated subject and the control subject, and a diminution of the intensity of infection in the treated subject as compared to the control subject identifies a successful protocol or drug.

[0013] In yet another aspect, the invention is directed to a method to target tumors using a therapeutic infective agent in a vertebrate subject comprising administering an infective agent that expresses a fluorescent protein to the vertebrate subject and observing the presence, absence, or intensity of fluorescence at various locations in the subject as a function of time.

Preferably the therapeutic infective agent targets the tumor and delivers a therapeutic product to the tumor.

[0014] The methods of the invention can also be used to monitor the nature of the microbial or viral systems that are significant in the progress of infection by coupling the nucleotide sequence encoding the fluorescent protein to various positions in the genome of the microbe or virus and monitoring the expression of the fluorescent protein by monitoring the fluorescence.

[0015] Lastly, the invention is directed to a tumor-targeting infective agent that expresses a fluorescent protein that is capable of targeting tumors in intact, living mammals in comparison to normal cells.

#### Brief Description of the Drawings

[0016] Figures 1A-1H show the locations of fluorescence in various parts of a mouse administered  $10^{11}$  *E. coli*-GFP by gavage. Figure 1A shows evidence of infection in the stomach immediately after gavage; Figures 1B-1G show the presence of fluorescence in the small intestine 10, 20, 30, 40, 50 and 60 minutes after gavage, respectively. Figure 1H shows the presence of infection in the colon 120 minutes after gavage.

[0017] Figures 2A-2C show the results of intravital imaging of *E. coli* after gavage with  $10^{11}$  *E. coli*-GFP. As shown in Figure 2A, GFP infection is present in the stomach and the duodenum immediately after gavage; Figure 2B shows the presence of infection in the small intestine 40 minutes after gavage; Figure 2C shows the presence of infection in the colon 120 minutes after gavage.

[0018] Figures 3A-3B show whole body and intravital imaging of infection in the stomach, small intestine and colon after gavage. Figure 3A shows a whole body image in the stomach (arrowhead), small intestine (fine arrows), and colon (thick arrow) after multiple gavage of aliquots of  $3 \times 10^{11}$  *E. coli*-GFP. Figure 3B shows corresponding intravital images labeled similarly.

[0019] Figure 4 shows the results of whole body imaging of infection in the colon immediately after enema of  $10^{11}$  *E. coli*-GFP.

[0020] Figures 5A-5D show the results of whole body imaging of peritoneal cavity infection in antibiotic response. Figures 5A and 5C show the infection in the peritoneal cavity immediately after intraperitoneal (i.p.) injection of  $10^9$  *E. coli*-GFP. Figure 5B shows an untreated mouse six hours after injection; the animal died at six hours. Figure 5D shows a Kanamycin treated mouse six hours after i.p. injection, wherein the animal survived.

[0021] Figure 6 shows the results of intravital imaging of intraperitoneal infection as described in Figure 5.

[0022] Figure 7A shows whole body imaging of an RFP-labeled U-87 human glioma growing in a nude mouse. Figure 7B shows fluorescence-guided injection of a PBS solution containing GFP-labeled *Salmonella*. Figure 7C shows whole body imaging of a GFP-labeled *Salmonella* in the RFP-labeled U-87 human glioma immediately after injection. Figure 7D shows the GFP-labeled *Salmonella* growing in the RFP-labeled U-87 human glioma one day after injection.

[0023] Figure 8A shows whole body imaging of an RFP-labeled DU-145 human prostate tumor in a nude mouse (Mouse 1). Figure 8B shows GFP-labeled *Salmonella* injected in the tumor of Mouse 1 imaged immediately after injection. Figure 8C shows whole body imaging of an RFP-labeled DU-145 human prostate tumor in a nude mouse (Mouse 2). Figure 8D shows the results of GFP-labeled *Salmonella* injected in the RFP-labeled DU-145 human prostate tumor which was imaged immediately after injection in Mouse 2.

[0024] Figure 9A shows whole body imaging of an RFP-labeled MDA MB-435 human breast tumor growing in a nude mouse. Figure 9B shows whole body imaging of GFP-labeled *Salmonella* injected in the tumor immediately after injection.

[0025] Figure 10A shows whole body imaging of a GFP-labeled PC-3 human prostate tumor growing in a nude mouse. Figure 10B shows the results of RFP-labeled *Salmonella*



injected in the tumor which was imaged immediately after injection. Figure 10C shows whole body imaging of an RFP-labeled *Salmonella* growing in the GFP-labeled PC-3 human prostate tumor one day after injection.

[0026] Figure 11A shows whole body imaging of a GFP-labeled PC-3 human prostate tumor growing in a nude mouse. Figure 11B shows the results of RFP-labeled *Salmonella* injected in the GFP-labeled PC-3 human prostate tumor immediately after injection. Figure 11C shows whole body imaging of an RFP-labeled *Salmonella* growing in the GFP-labeled PC-3 human prostate tumor one day after injection. Figure 11D shows whole body imaging of an RFP-labeled *Salmonella* growing in the GFP-labeled PC-3 human prostate tumor four days after injection.

[0027] Figure 12A shows whole body imaging of a GFP-labeled PC-3 human prostate tumor growing in a nude mouse. Figure 12B shows the results of RFP-labeled *Salmonella* injected in the GFP-labeled PC-3 human prostate tumor which was imaged immediately after injection. Figure 12C shows whole body imaging of an RFP-labeled *Salmonella* growing in the GFP-labeled PC-3 human prostate tumor one day after injection. Figure 12D shows whole body imaging of an RFP-labeled *Salmonella* growing in the GFP-labeled PC-3 human prostate tumor four days after injection.

[0028] Figure 13 shows RFP-labeled *Salmonella* targeting and progressively growing in GFP-labeled PC-3 human prostate tumor growing in nude mice demonstrated by histology. RFP-labeled *Salmonella* growing in the GFP-labeled PC-3 human prostate tumor four days after injection (Figure 12D).

[0029] Figures 14A-14B show the effect of treatment of RFP-labeled *Salmonella* on PC-3 human prostate tumor growing in nude mice demonstrated by histology. Figure 14A is the untreated control. Figure 14B is the treatment after RFP-labeled *Salmonella*.

#### Modes of Carrying Out the Invention

[0030] The invention provides model systems for the study of the mechanism of infection. Advantage is taken of visible marker fluorescence proteins to label the infectious agents so that their migration and colonization in tissues can be followed as the infection progresses.

[0031] As used herein, "progression of infection" refers to the general time-dependent manner in which infective agent and infected cells migrate and/or proliferate through an infected

organism. The progress of infection may be a function simply of the location of the infectious agent or infected cells but generally also is a function of the proliferation of the infective agent and infected cells. Thus, both the location and intensity of fluorescence are significant in monitoring progression.

5       [0032] Since sufficient intensity can be achieved to observe the migration of fluorescent cells in the intact animal, in addition to determining the migration of the infectious agent by excising organs or tissue, if desired, the progression of metastasis can be observed in the intact subject. Either or both methods may be employed to observe the progress of infection and in evaluating, in model systems, the efficacy of potential protocols and drugs.

10       [0033] In addition, the present invention takes advantage of delivering therapeutics by infective agents to tumors and provides an accurate tumor targeting mechanism. It is advantageous in tumor targeting to be able to visualize the infective agent as well as its therapeutic molecule. Some advantages of fluorescence guided injection of tumors are that there is no lower limit to the size of tumor that can be treated, and further, the method is independent  
15 of tumor necrosis. In addition, infective agents are not limited to anaerobes nor non-virulent strains of infective agents. A "therapeutic," "therapeutic molecule" or "therapeutic product" as used herein refers to a gene of interest that is contained in an infective agent, or a product secreted from the infective agent, such as a toxin or other therapeutic protein, or a product that is not secreted but which is used by the infective agent such that a therapeutic effect on tumor is  
20 affected. A gene of interest means any gene that has a therapeutic effect on tumor such as a gene that expresses an anti-tumor agent. Examples of a therapeutic molecule is a gene expressing methioninase or methioninase itself as disclosed in U.S. Pat. No. 6,231,854. Other examples include p53, BAX, toxins, tumor necrosis factor (TNF), TNF-related apoptosis-inducing ligand, Fas ligand, and antibodies against death receptors.

25       [0034] The label used in the various aspects of the invention is a fluorescent protein. The native gene encoding the seminal protein in this class, green fluorescent protein (GFP) has been cloned from the bioluminescent jellyfish *Aequorea victoria* (Morin, J., *et al.*, *J. Cell Physiol* (1972) 77:313-318). The availability of the gene has made it possible to use GFP as a marker for gene expression. The original GFP itself is a 283 amino acid protein with a molecular  
30 weight of 27 kD. It requires no additional proteins from its native source nor does it require substrates or cofactors available only in its native source in order to fluoresce. (Prasher, D.C., *et*

al., *Gene* (1992) 111:229-233; Yang, F., et al., *Nature Biotechnol* (1996) 14:1252-1256; Cody, C.W., et al., *Biochemistry* (1993) 32:1212-1218.) Mutants of the original GFP gene have been found useful to enhance expression and to modify excitation and fluorescence, so that "GFP" in various colors, including reds and blues has been obtained. GFP-S65T (wherein serine at 65 is replaced with threonine) is particularly useful in the present invention method and has a single excitation peak at 490 nm. (Heim, R., et al., *Nature* (1995) 373:663-664); U.S. Patent No. 5,625,048. Other mutants have also been disclosed by Delagrade, S., et al., *Biotechnology* (1995) 13:151-154; Cormack, B., et al., *Gene* (1996) 173:33-38 and Cramer, A., et al., *Nature Biotechnol* (1996) 14:315-319. Additional mutants are also disclosed in U.S. Patent No. 5,625,048. By suitable modification, the spectrum of light emitted by the GFP can be altered. Thus, although the term "GFP" is often used in the present application, the proteins included within this definition are not necessarily green in appearance. Various forms of GFP exhibit colors other than green and these, too, are included within the definition of "GFP" and are useful in the methods and materials of the invention. In addition, it is noted that green fluorescent proteins falling within the definition of "GFP" herein have been isolated from other organisms, such as the sea pansy, *Renilla reniformis*. Any suitable and convenient form of GFP can be used to modify the infectious agents useful in the invention, both native and mutated forms.

[0035] In order to avoid confusion, the simple term "fluorescent protein" will be used; in general, this is understood to refer to the fluorescent proteins which are produced by various organisms, such as *Renilla* and *Aequorea* as well as modified forms of these native fluorescent proteins which may fluoresce in various visible colors, such as red, yellow, and cobalt, which are exhibited by red fluorescent protein (RFP), yellow fluorescent protein (YFP) or cobalt fluorescent protein (CFP), respectively. In general, the terms "fluorescent protein" and "GFP" or "RFP" are used interchangeably.

[0036] Because fluorescent proteins are available in a variety of colors, imaging with respect to more than a single color can be done simultaneously. For example, two different infective agents or three different infective agents each expressing a characteristic fluorescence can be administered to the organism and differential effects of proposed treatments evaluated. In addition, a single infectious organism could be labeled constitutively with a single color and a different color used to produce a fusion with a gene product either intracellular or that is

secreted. Thus, the nucleotide sequence encoding a fluorescent protein having a color different from that used to label the organism *per se* can be inserted at a locus to be studied or as a fusion protein in a vector with a protein to be studied. As a further illustration, toxins and other potentially therapeutic proteins will be genetically linked with RFP in order to label and visualize the therapeutic product of GFP-labeled bacteria and visa versa. Two-color imaging will be used to visualize targeting of the bacteria to the tumor as well as their secreted therapeutic product. These tumor-targeting bacteria will be adapted for selective growth in tumors as visualized by their fluorescence. Further, one or more infective agents could each be labeled with a single color, a gene of interest with another color, and the tumor with a third color. For example, fluorescence-expressing tumors in laboratory animals will enable visualization of tumor targeting of fluorescence-labeled infective agents by whole body imaging, as well as the infective agents' therapeutic product.

[0037] As exemplified herein, GFP-and RFP-labeled bacteria were delivered by fluorescence-guided injection in GFP- and RFP-labeled tumors implanted in nude mice and thus the bacteria was targeted to GFP-labeled tumor, thereby inducing tumor necrosis. In particular, the targeting of GFP-and RFP-labeled *E. coli* and *S. typhimurium* to RFP- and- GFP- expressing tumors in mice was visualized by dual-color whole-body imaging. GFP-and RFP-labeled bacteria growing in targeted RFP-and GFP- labeled tumors have been visualized by dual-color whole-body imaging as shown in the Examples herein. Thus, tumor targeting of fluorescent labeled microorganisms has been shown. The method of the invention can also be used, however, to monitor the mis-targeting of the infective agent in order ultimately to select for bacteria that targets tumors.

[0038] Techniques for labeling cells in general using GFP are disclosed in U.S. 5,491,084 (*supra*).

[0039] The methods of the invention utilize infectious agents which have been modified to express the nucleotide sequence encoding a fluorescent protein, preferably of sufficient fluorescence intensity that the fluorescence can be seen in the subject without the necessity of any invasive technique. While whole body imaging is preferred because of the possibility of real-time observation, endoscopic techniques, for example, can also be employed or, if desired, tissues or organs excised for direct or histochemical observation.

[0040] The nucleotide sequence encoding the fluorescent protein may be introduced into the infectious agent by direct modification, such as modification of a viral genome to locate the fluorescent protein encoding sequence in a suitable position under the control sequences endogenous to the virus, or may be introduced into microbial systems using appropriate expression vectors. Infective agents may be bacteria, eukaryotes such as yeast, protozoans such as malaria, or viruses. A multiplicity of expression vectors for particular types of bacterial, protozoan, and eukaryotic microbial systems is well known in the art. A litany of control sequences operable in these systems is by this time well understood. The infectious agent is thus initially modified either to express the fluorescent protein under control of a constitutive promoter as a constant feature of cell growth and reproduction, or may be placed in the microbial or viral genome at particular desired locations, replacing endogenous sequences which may be involved in virulence or otherwise in the progress of infection to study the temporal and spatial parameters characteristic of expression of these endogenous genes. Thus, it is possible to explore the types of factors endogenous to the microbe or virus which contribute to the effectiveness of the infection by suitable choice of positioning. Similarly, a gene expressing a fluorescent protein may be introduced into tumor cells such that laboratory animals contain tumors that can be visualized. Another approach to prepare fluorescent tumors is through photodynamic therapy (PDT) where the tumor absorbs agents that fluoresce such as clinically approved agents, for example, hematoporphorins

[0041] The appropriately modified infectious agent is then administered to the subject in a manner which mimics, if desired, the route of infection believed used by the agent or by an arbitrary route. Administration may be by injection, gavage, oral, by aerosol into the respiratory system, by suppository, by contact with a mucosal surface in general, or by any suitable means known in the art to introduce infectious agents. In tumor targeting where the tumor expresses a fluorescent protein, administration can be made by fluorescent guided injection. Unlike the situation with regard to the study of tumor metastasis using fluorescence, it is not necessary that the subject be immunocompromised since infection occurs readily in organisms with intact immune systems. However, immunocompromised subjects may also be useful in studying the progress of the condition.

[0042] Although endoscopy can be used as well as excision of individual tissues, it is particularly convenient to visualize the migration of infective agent and infected cells in the

intact animal through fluorescent optical tumor imaging (FOTI). This permits real-time observation and monitoring of progression of infection on a continuous basis, in particular, in model systems, in evaluation of potential anti-infective drugs and protocols. Thus, the inhibition of infection observed directly in test animals administered a candidate drug or protocol in comparison to controls which have not been administered the drug or protocol indicates the efficacy of the candidate and its potential as a treatment. In subjects being treated for infection, the availability of FOTI permits those devising treatment protocols to be informed on a continuous basis of the advisability of modifying or not modifying the protocol. In one embodiment, to ascertain the feasibility of fluorescently-labeled bacteria to target tumors, GFP-labeled bacteria were injected into the Lewis lung tumor growing in nude mice. The tumor area became highly fluorescent and readily visualized by blue light excitation in a light box with a CCD camera and a GFP filter.

[0043] Suitable vertebrate subjects for use as models are preferably mammalian subjects, most preferably convenient laboratory animals such as rabbits, rats, mice, and the like. For closer analogy to human subjects, primates could also be used. Any appropriate vertebrate subject can be used, the choice being dictated mainly by convenience and similarity to the system of ultimate interest. Ultimately, the vertebrate subjects can be humans.

[0044] It is expected that tumor-targeting bacteria can be adapted for selective growth in tumors as vectors for tumor-selective gene therapy.

[0045] The following examples are intended to illustrate but not to limit the invention.

#### Preparation A

##### Modification of Infectious Agents

[0046] A variant of the *Renilla mulleri* green fluorescent protein (RMV-GFP) (Zhao, M., Xu, M., Hoffman, R.M., unpublished data) was cloned into the BamHI and NotI sites of the pUC19 derivative pPD16.38 (Clontech, Palo Alto, CA) with GFP expressed from the *lac* promoter. The vector was termed pRMV-GFP. pRMV-GFP was transfected into *E. coli* JM 109 competent cells (Stratagene, San Diego, CA) by standard methods, and transformed cells were selected by ampicillin resistance on agar plates. High expression *E. coli*-GFP clones were selected by fluorescence microscopy.

[0047] *E. coli* has also been labeled with RFP and, in addition, *Salmonella typhimurium* has been labeled with both the GFP and RFP.

#### Example 1

##### Infection of Mice by Gavage

5 [0048] *Nu/nu*/CD-1 mice, 4 weeks old, female, mice were gavaged with 0.5 ml of an *E. coli*-GFP suspension ( $5 \times 10^{10}$ /ml) with a 20 gauge barrel tip feeding needle (Fine Science Tools Inc., Foster City, CA) and latex-free syringe (Becton Dickinson, Franklin Lakes, NJ).

[0049] After gavage, at various time points, imaging of the mice was performed. Imaging was carried out in a light box illuminated by blue light fiber optics (Lighttools Research, Inc.,  
10 Encinitas, CA). Images were captured using a Hamamatsu C5810 3-chip cooled color CCD camera (Hamamatsu Photonics Systems, Bridgewater, NJ). Images of 1024 x 724 pixels were captured directly on an IBM PC or continuously through video output on a high resolution Sony VCR model SLV-R1000 (Sony Corp., Tokyo, Japan). Images were processed for contrast and brightness and analyzed with the use of Image Pro Plus 3.1 software (Media Cybernetics, Silver  
15 Springs, MD).

[0050] *E. coli*-GFP introduced to the mouse GI tract by gavage became visible in the stomach in whole body images almost immediately (Figure 1A). The stomach emptied within 10 minutes post gavage and the *E. coli*-GFP next appeared in the small intestine (Figure 1B-G). The bacterial population in the small intestine appeared to peak at 40 minutes post gavage  
20 (Figure 1E) and disappeared by 120 minutes (Figure 1G). After 120 minutes, *E. coli*-GFP appeared in the colon (Figure 1H).

[0051] At appropriate times after gavage, the abdominal cavity was opened and intravital images made of the *E. Coli*-GFP fluorescence. The stomach (Figure 2A), small intestine (Figure 2B), and colon (Figure 2C) were brightly fluorescent with *E. coli*-GFP as seen by  
25 intravital imaging. Multiple gavage with *E. coli*-GFP allowed simultaneous inoculation of the stomach, small intestine, and colon, which were imaged by whole-body (Figure 3A) and intravital techniques (Figure 3B). Comparison of whole-body and intravital images of *E. coli*-GFP in the stomach, small intestine, and colon showed a high degree of correspondence.

### Example 2

#### *E. coli*-GFP Direct Colon Infection

[0052] One and one half ml containing  $3 \times 10^{10}$  *E. coli*-GFP per mouse were administered into the colon by enema using a 20 gauge barrel tip feeding needle (Fine Science Tools Inc., Foster City, CA) and latex-free syringe (Becton Dickinson). These mice were also subjected to imaging using the techniques of Example 1. The results are shown in Figure 4.

### Example 3

#### *E. coli*-GFP Peritoneal Infection and Response to Antibiotics

[0053] The mice in each group were given an intraperitoneal (i.p.) injection of  $10^9$ - $10^{10}$  *E. coli*-GFP using a 1 ml 29G1 latex-free syringe (Becton Dickinson). Immediately after injection, the fluorescent bacteria were seen localized around the injection site by external whole-body imaging. (Figure 5A, C). Six hours later, the *E. coli*-GFP were seen to spread throughout the peritoneum (Figure 5B), coinciding with the death of the animal. Intravital imaging of *E. coli*-GFP in the open peritoneal cavity at 6 hours (Figure 6) showed a bacterial distribution similar to that seen by external whole-body imaging.

[0054] Another group of intraperitoneally infected animals were treated with 2 mg Kanamycin in 100  $\mu$ l following inoculation. A control group of infected mice were given an i.p. injection of 100  $\mu$ l of PBS instead of antibiotic. Whole-body imaging of treated mice showed a marked reduction of the bacterial population over the next six hours (Figure 5C, D).

### Example 4

#### Targeting in Brain Cancer Using Whole-Body Imaging

[0055] A PBS solution (10  $\mu$ l) containing  $1 \times 10^8$  GFP-labeled *Salmonella typhimurium* was injected in the RFP-labeled U-87 human glioma in a nude mouse (Figure 7A) using fluorescence guided injection (Figure 7B). GFP-labeled *Salmonella* in the RFP-labeled U-87 human glioma was imaged using techniques similar to Example 1 immediately after injection (Figure 7C). GFP-labeled *Salmonella* growing in the RFP-labeled U-87 human glioma one day after injection was seen showing GFP-labeled *Salmonella* localization around the tumor as well as reduction of tumor size (Figure 7D).



Example 5Targeting in Prostate Tumors Using Whole-Body Imaging

[0056] In a first nude mouse having an RFP-labeled DU-145 human prostate tumor (Figure 8A),  $1 \times 10^8$  GFP-labeled *Salmonella typhimurium* was injected in the RFP-labeled DU-145 human prostate tumor and imaged using whole-body imaging immediately after injection (Figure 8B). GFP-labeled *Salmonella* localization around the tumor (Figure 8B) was seen. One advantage to fluorescent guided injection is that virulent *Salmonella* can be used and tumors of all sizes can be targeted.

[0057] In a second nude mouse having an RFP-labeled DU-145 human prostate tumor (Figure 8C), a solution containing  $2 \times 10^8$  GFP-labeled *Salmonella typhimurium* was injected in the RFP-labeled DU-145 human prostate tumor and imaged using whole-body imaging immediately after injection. GFP-labeled *Salmonella typhimurium* localization in the tumor (Figure 8D) was seen.

Example 6Targeting in Breast Cancer Using Whole-Body Imaging

[0058] A solution containing  $2 \times 10^8$  GFP-labeled *Salmonella typhimurium* was injected in the RFP-labeled MDA MB-435 human breast tumor growing in a nude mouse (Figure 9A) and imaged using techniques similar to Example 1 immediately after injection showing localization around the tumor (Figure 9B) and apparent reduction of tumor size, indicating tumor necrosis.

Example 7Targeting in Prostate Tumor Using Whole-Body Imaging

[0059] A solution containing  $3 \times 10^8$  RFP-labeled *Salmonella typhimurium* was injected in the GFP-labeled PC-3 human prostate tumor growing in a nude mouse (Figure 10A) and imaged using techniques similar to Example 1 immediately after injection (Figure 10B). Growth of RFP-labeled *Salmonella typhimurium* in the GFP-labeled PC-3 human prostate tumor was seen one day after injection (Figure 10C) showing RFP-labeled *Salmonella* growth around the tumor and reduction of tumor size.

Example 8Targeting in Prostate Tumor Using Whole-Body Imaging

[0060] A solution containing  $2 \times 10^8$  RFP-labeled *Salmonella typhimurium* was injected in the GFP-labeled PC-3 human prostate tumor growing in a nude mouse (Figure 11A) and imaged using techniques similar to Example 1 immediately after injection (Figure 11B). RFP-labeled *Salmonella* was detected as growing in the GFP-labeled PC-3 human prostate tumor one day after injection (Figure 11C) and continuing to grow in the tumor four days after injection (Figure 11D) while reduction of tumor size is shown.

Example 9Targeting in Prostate Tumor Using Whole-Body Imaging

[0061] A solution containing  $2 \times 10^8$  RFP-labeled *Salmonella typhimurium* was injected in the GFP-labeled PC-3 human prostate tumor growing in a nude mouse (Figure 12A) and imaged using techniques similar to Example 1 immediately after injection (Figure 12B). RFP-labeled *Salmonella* is seen growing in the GFP-labeled PC-3 human prostate tumor one day after injection (Figure 12C) and four days after injection (Figure 12D) showing visible reduction in tumor size.

[0062] Histological studies were performed on the RFP-labeled *Salmonella* growing in the GFP-labeled PC-3 human prostate tumor four days after injection (Figure 12D) by fixing the tumor tissue with 10% buffered formaline and processed for paraffin section and HE staining by standard method. The RFP-labeled *Salmonella* (the small blue dots as pointed by the white arrows in Figure 13, Magnification 400X) were progressively growing in the PC-3 tumor tissue and targeting the tumor cells.

[0063] Histological studies (HE staining, Magnification 200X) also compared an untreated control showing a well-maintained PC-3 human prostate tumor structure growing in a nude mouse (Figure 14A) with PC-3 human prostate tumor growing in a nude mouse treated with RFP-labeled *Salmonella* four days after injection (Figure 14B). The majority of tumor tissue has been destroyed, and the extensive necrosis (arrows in Figure 14B) in the tumor is shown.

Claims

1. A method to monitor the progression of infection in a vertebrate subject which method comprises

observing the presence, absence or intensity of fluorescence at various locations in said  
5 subject as a function of time, wherein said vertebrate subject has been treated with an infective  
agent that expresses a fluorescent protein.

2. The method of claim 1, wherein said observing is by endoscopy or fluorescent  
optical tumor imaging in the intact subject.

3. The method of claim 1, wherein the fluorescent protein has green fluorescence or  
10 red fluorescence.

4. The method of claim 1, wherein the infective agent is a bacterium, protozoan or  
virus.

5. The method of claim 1, wherein the infective agent is a eukaryotic single-celled  
organism.

6. The method of claim 1, wherein the subject is a mammal.

7. The method of claim 6, wherein the subject is a mouse, rat or rabbit.

8. The method of claim 1, wherein the subject is immunocompromised.

9. A method to evaluate a candidate protocol or drug for the inhibition of infection  
which method comprises

administering said protocol or drug to a vertebrate subject which has been treated with  
an infective agent that expresses a fluorescent protein and monitoring the progression of  
infection over time by observing the presence, absence or intensity of the fluorescence at various  
20 locations in said treated subject;

monitoring the progression of infection over time in a control subject which has been similarly treated with an infective agent that expresses a fluorescent protein; and  
comparing the progression of infection in said treated subject with the progression of infection in said control subject;

5 whereby a diminution of the progression of infection in said treated subject as compared to said control subject identifies the protocol or drug as effective in inhibiting infection.

10. The method of claim 9, wherein said monitoring is by endoscopy or fluorescent optical tumor imagining in the intact subject.

10 11. The method of claim 9, wherein the infective agent is a bacterium, protozoan or virus.

12. The method of claim 9, wherein the infective agent is a eukaryotic single-celled organism.

13. The method of claim 9, wherein the subject is a mammal.

14. The method of claim 13, wherein the subject is a mouse, rat or rabbit.

15 15. The method of claim 9, wherein the subject is immunocompromised.

20 16. A method to identify genes associated with infection, which method comprises observing the presence, absence or intensity of fluorescence in said subject as a function of time and location in said subject, wherein said subject has been infected with an infective agent with an altered genome, wherein the alteration in the genome comprises replacement of a nucleotide sequence whose function is to be determined with a nucleotide sequence encoding a fluorescent protein.

17. The method of claim 16, wherein the infective agent is a bacterium, protozoan or virus.

18. The method of claim 16, wherein the infective agent is a eukaryotic single-celled organism.

19. The method of claim 16, wherein the subject is a mammal.

20. The method of claim 19, wherein the subject is a mouse, rat or rabbit.

5 21. The method of claim 16, wherein the subject is immunocompromised.

22. The method of claim 16, wherein said observing is by endoscopy or fluorescent optical tumor imaging in the intact subject.

23. A method of targeting tumors using a therapeutic infective agent in a vertebrate subject comprising

10 administering an infective agent that expresses a fluorescent protein to said vertebrate subject containing a tumor; and

observing the presence, absence or intensity of fluorescence in said subject as a function of time.

15 24. The method of claim 23, wherein the therapeutic infective agent delivers a therapeutic product to the tumor.

25. The method of claim 23, wherein the tumor exhibits fluorescence of a color other than the color of the infective agent.

26. The method of claim 24, wherein the therapeutic product exhibits fluorescence of a color different than the color of the infective agent and the tumor.

20 27. The method of claim 23, wherein the infective agent is a bacterium, a protozoan or a virus.

28. The method of claim 23, wherein the infective agent is a eukaryotic single-celled organism.

29. The method of claim 23, wherein the subject is a mammal.

30. The method of claim 29, wherein the subject is a mouse, rat or rabbit.

5 31. The method of claim 22, wherein the subject is immunocompromised.

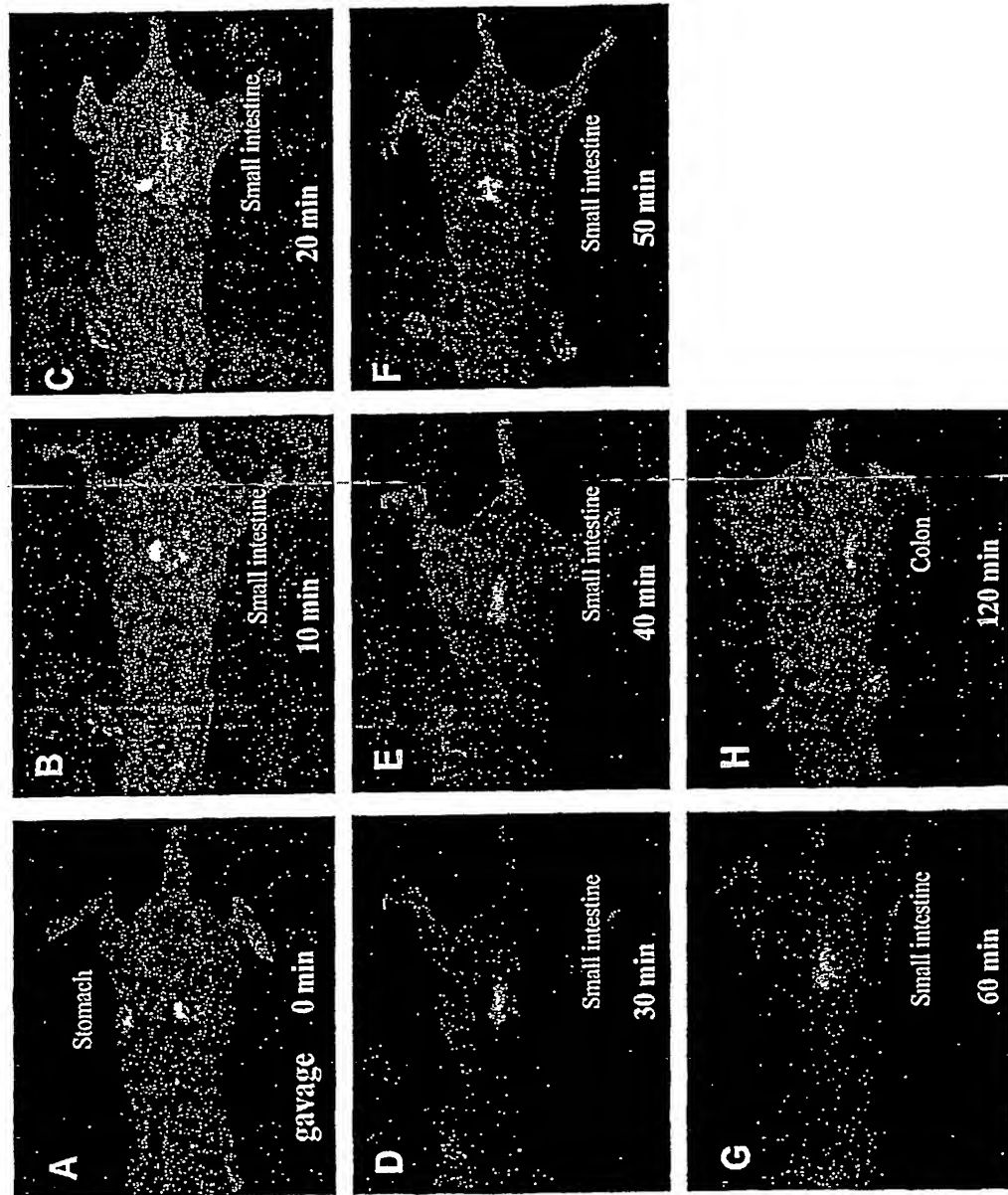
32. The method of claim 22, wherein said observing is by endoscopy or by fluorescent optical tumor imaging in the intact subject.

33. The method of claim 23, wherein tumor necrosis is induced.

10 34. A tumor targeting infective agent comprising an infective agent that expresses a fluorescent protein, where the infective agent is capable of preferentially targeting the tumor in an intact, living mammal in comparison to normal cells.

35. A tumor targeting infective agent as in claim 34, wherein the infective agent contains or secretes a therapeutic molecule or contains a gene of interest.

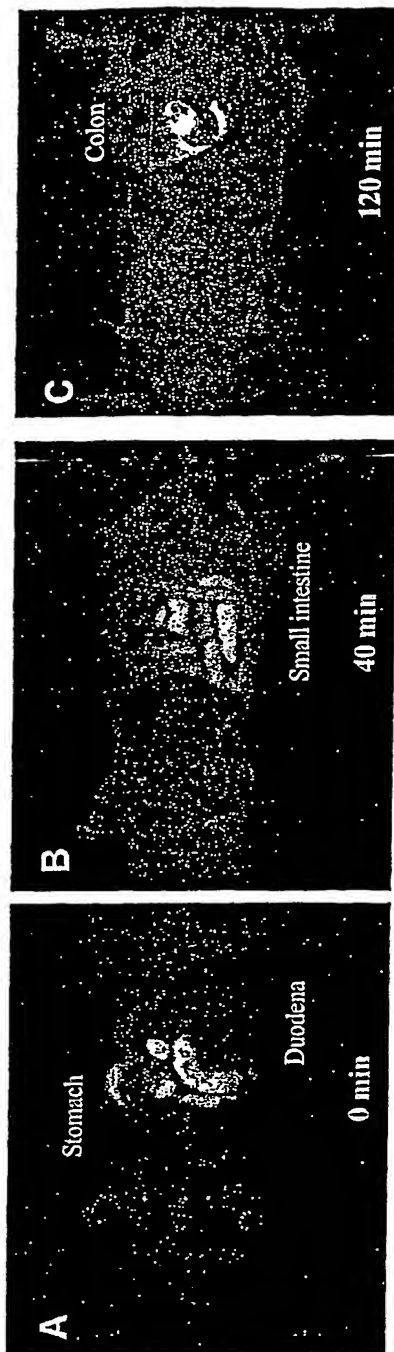
Fig. 1  
Whole-body imaging of *E. coli*-GFP infection in various organs



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Fig. 2

Intra-vital imaging of *E. coli*-GFP infection in the stomach, small intestine and colon after gavage

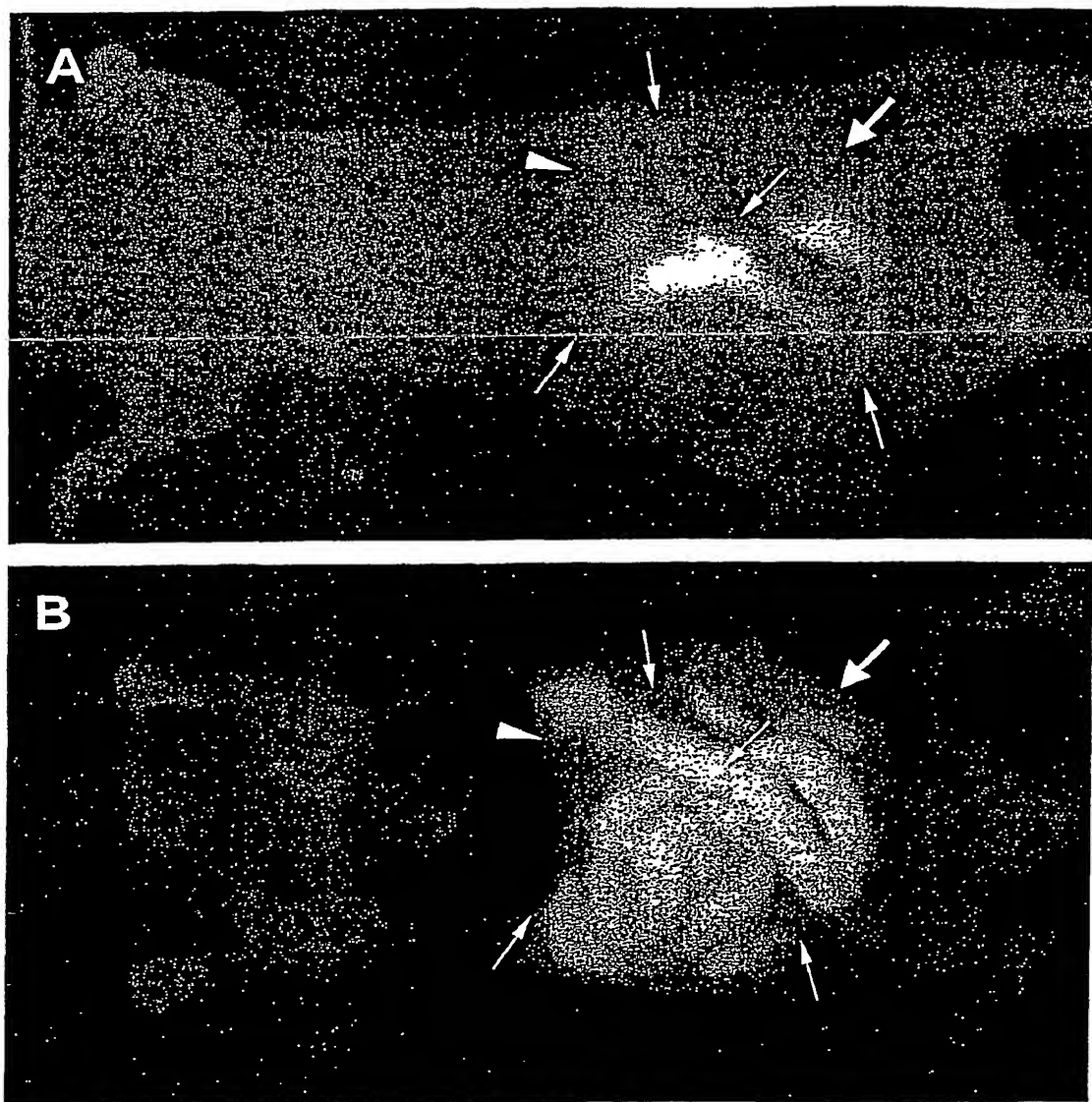




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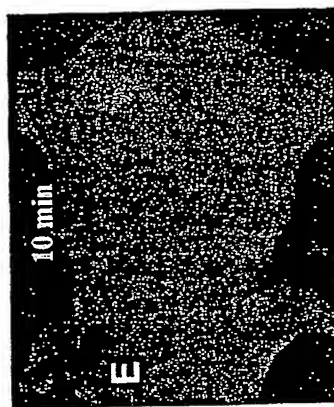
Fig. 3

Whole-body and intra-vital imaging of *E. coli*-GFP infection in the stomach, small intestine and colon after gavage



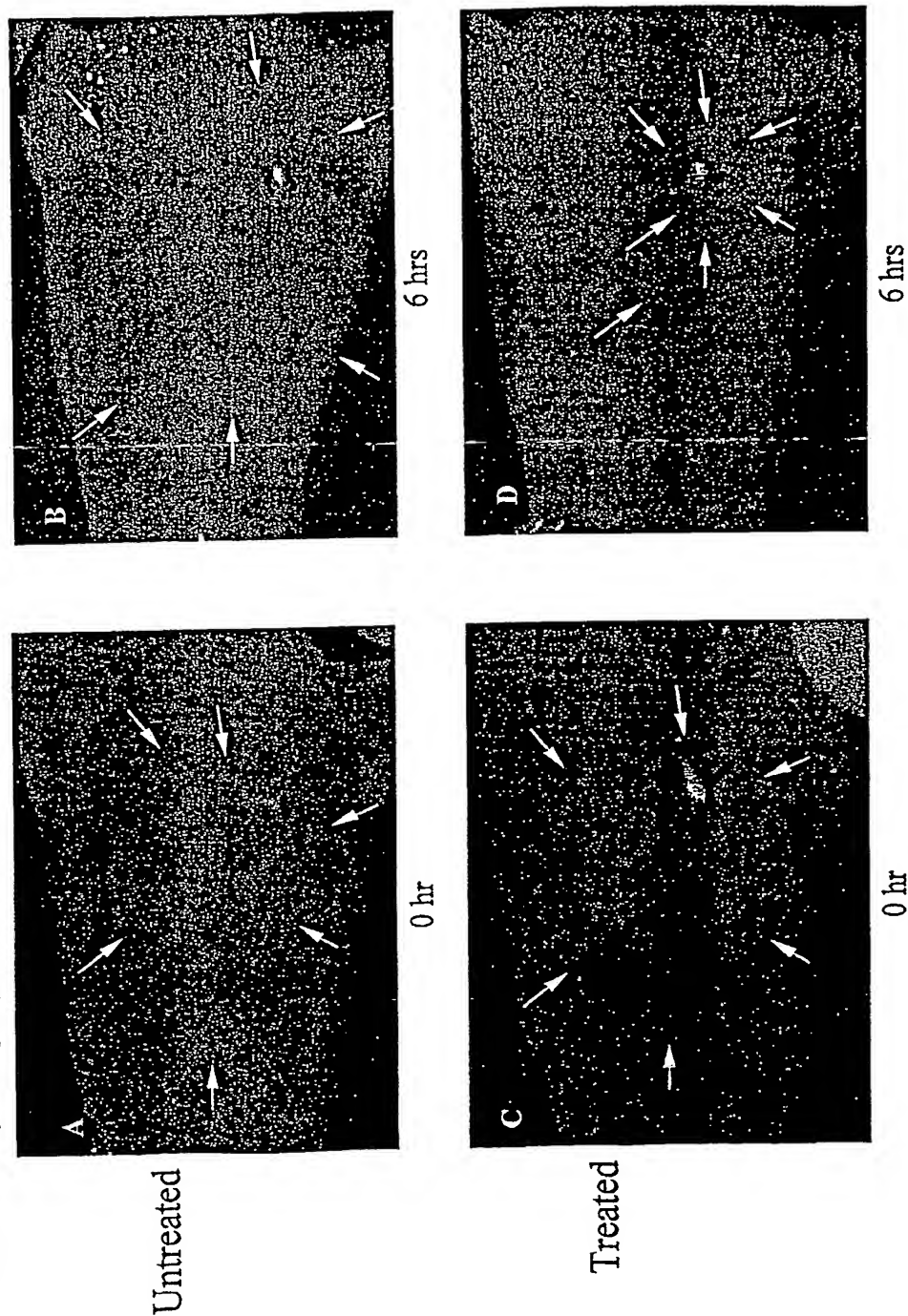
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Fig. 4  
Whole-body imaging of *E. coli*-GFP infection in the colon



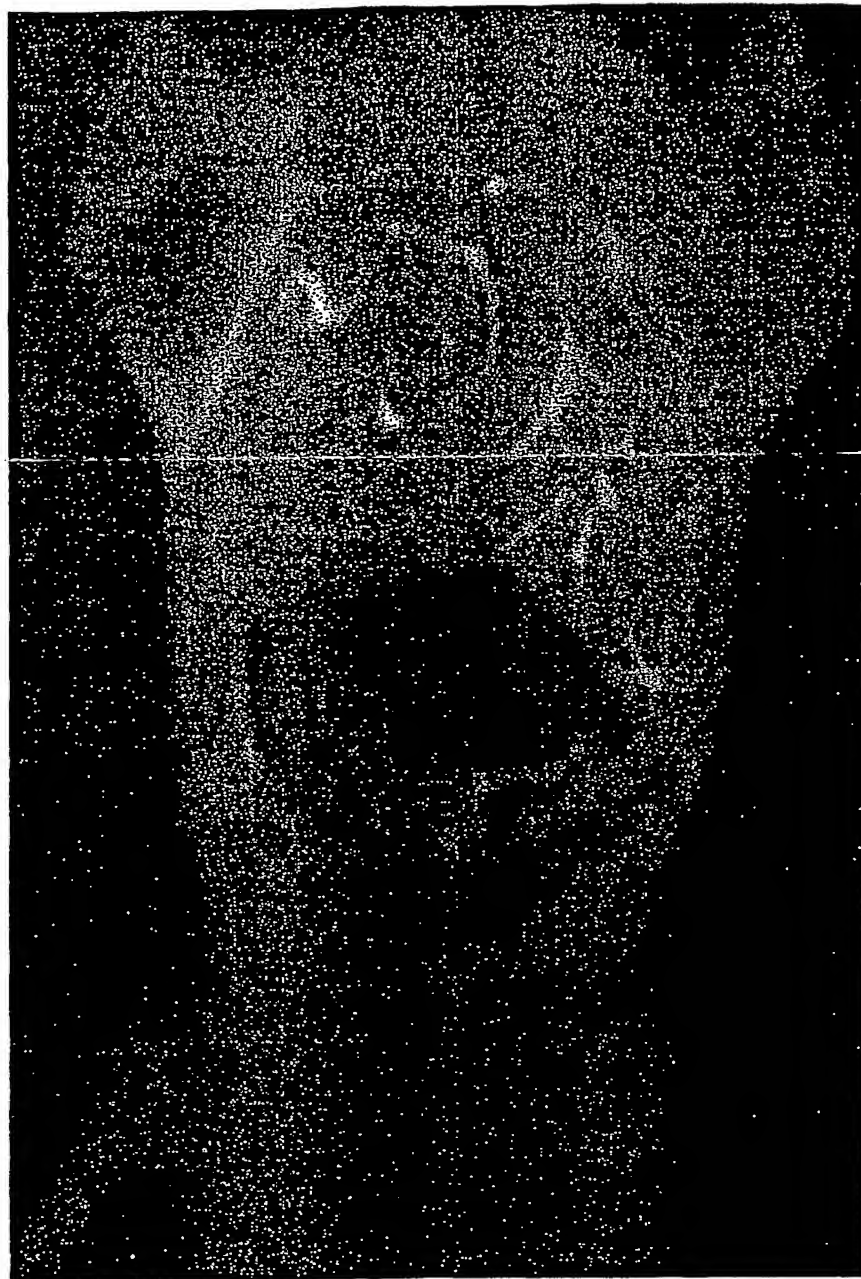
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Fig. 5  
Whole-body imaging of *E. coli*-GFP peritoneal cavity infection and antibiotic response

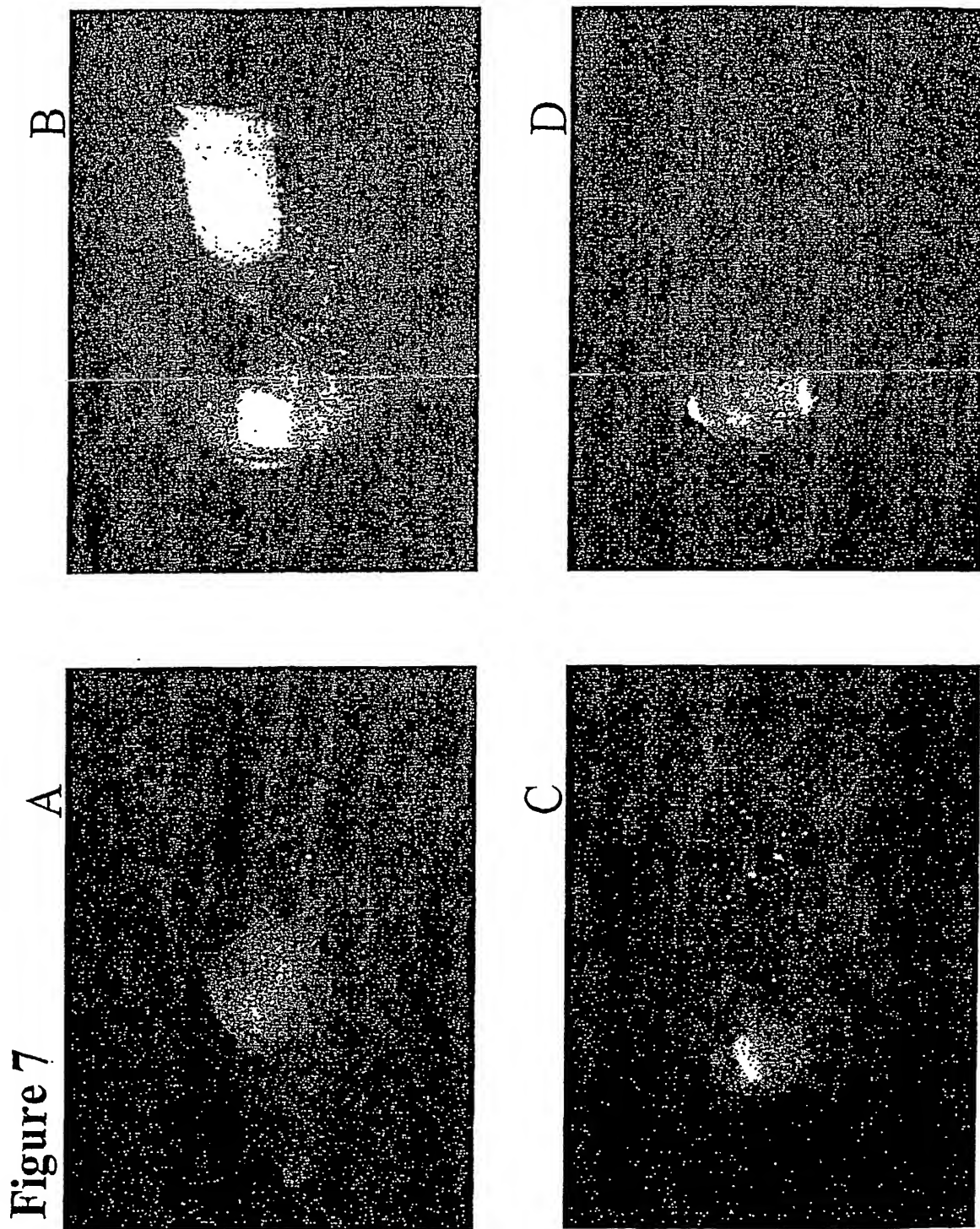


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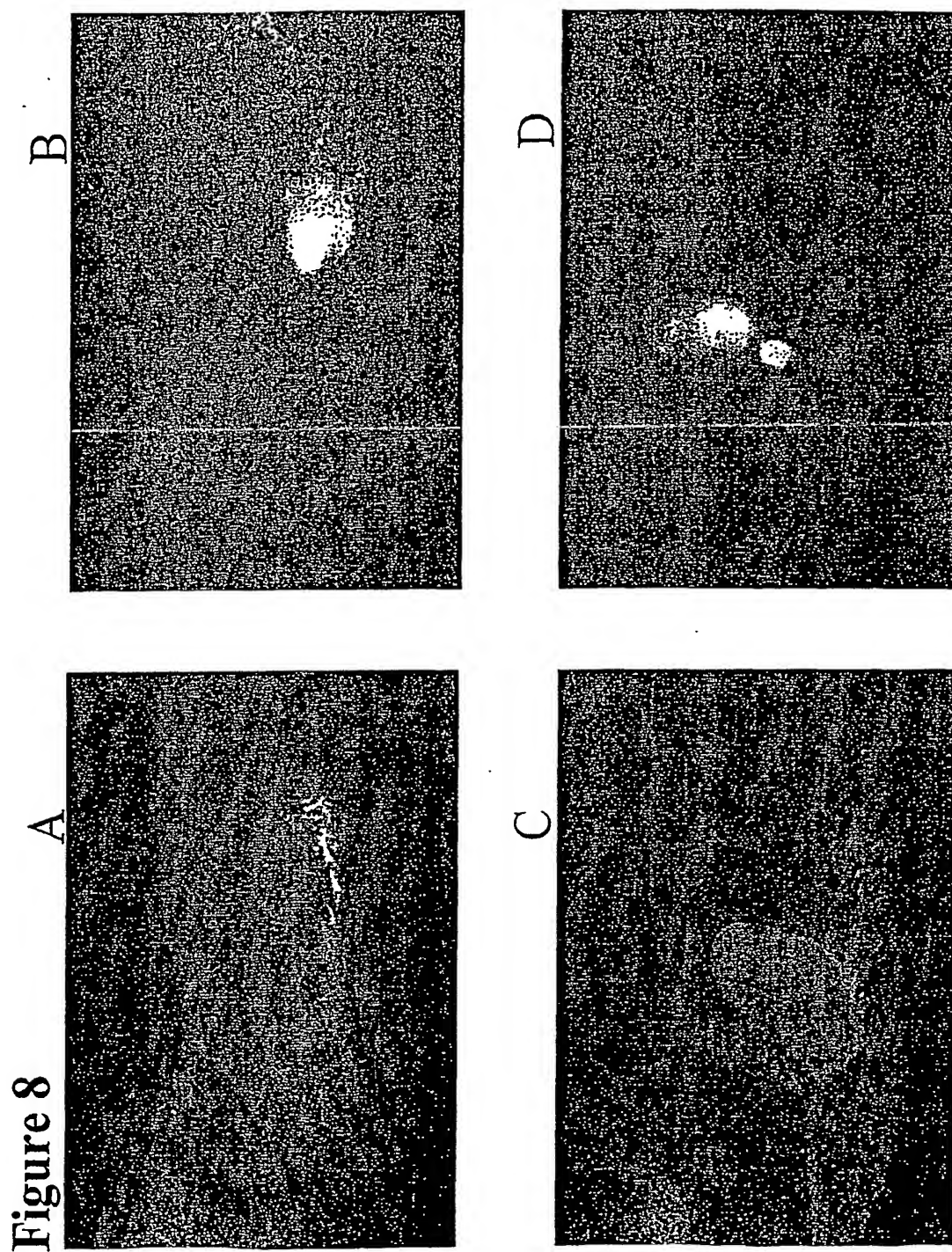
Fig. 6  
Intra-vital imaging of *E. coli*-GFP peritoneal cavity infection



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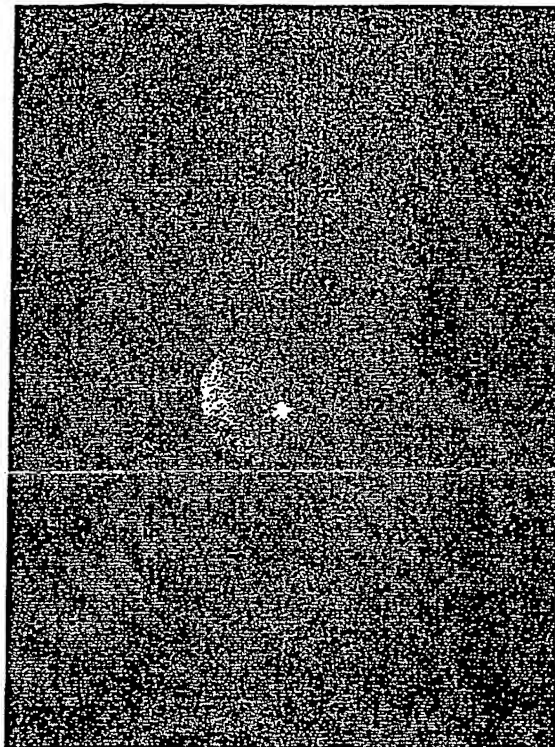


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B



A

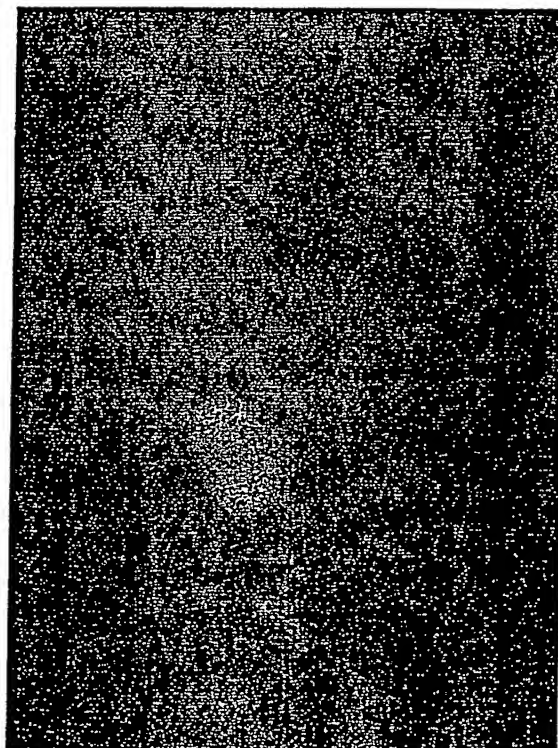
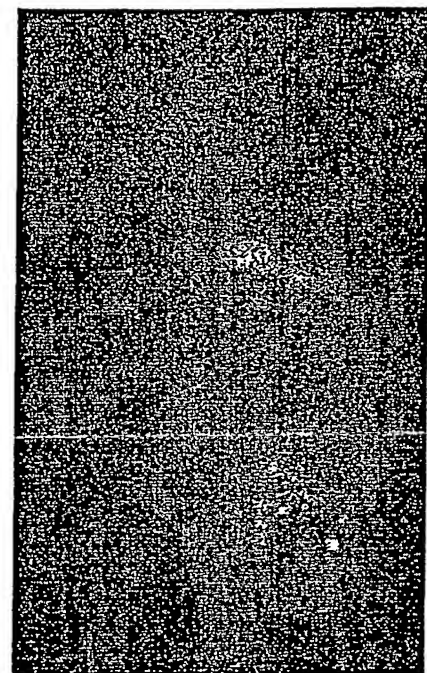


Figure 9

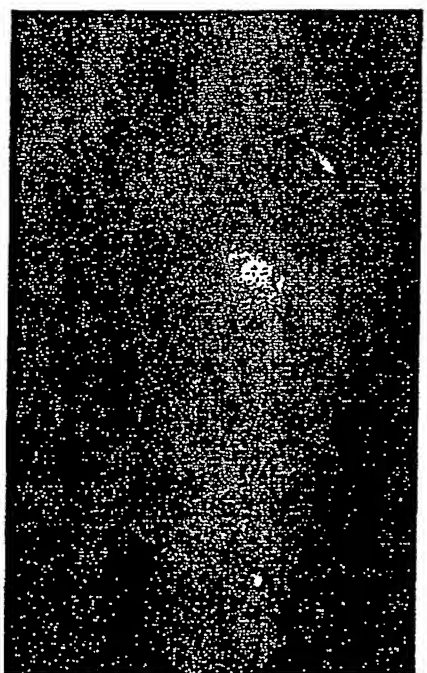


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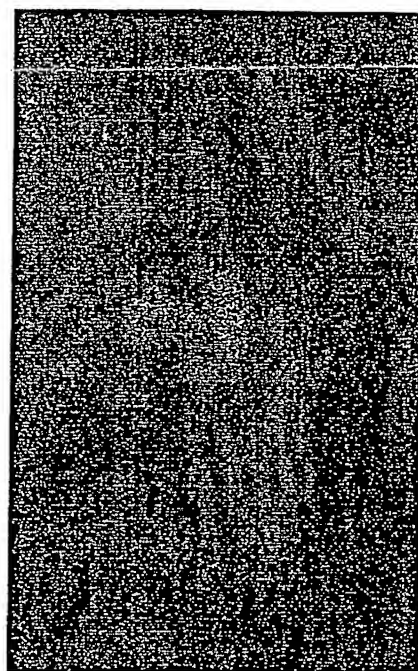
Figure 10



B



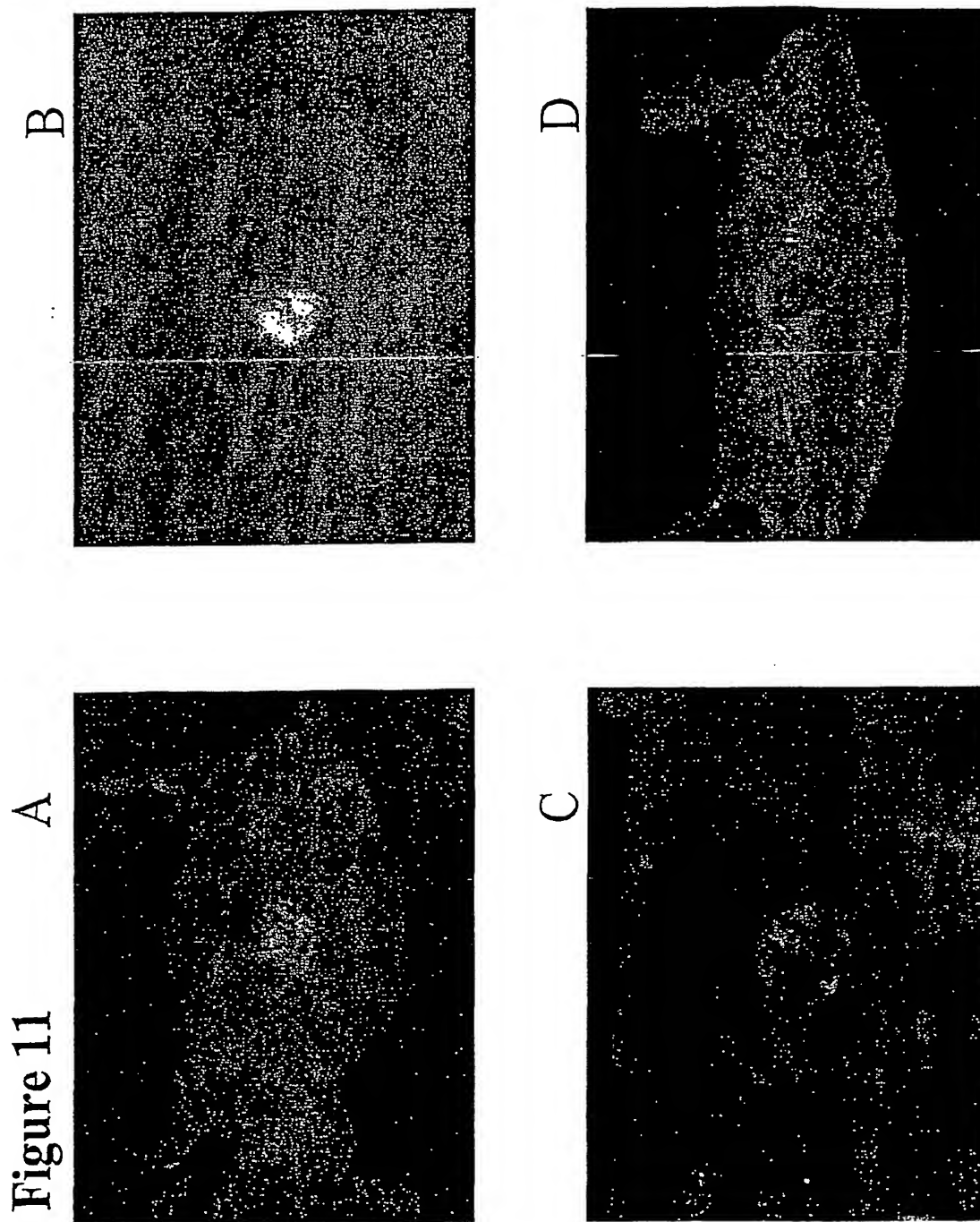
A



C

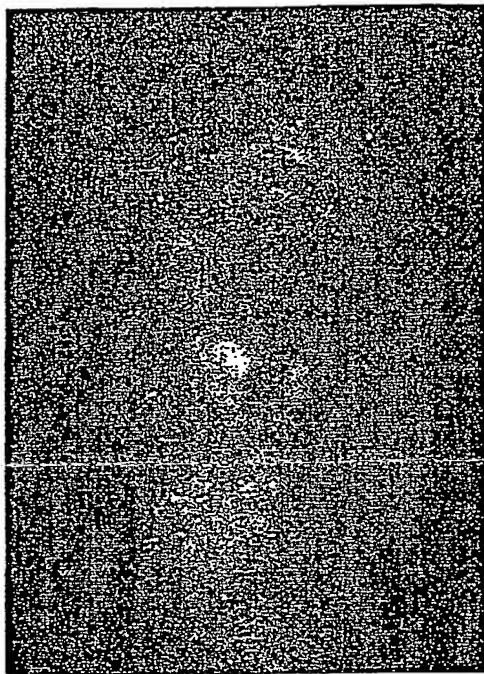


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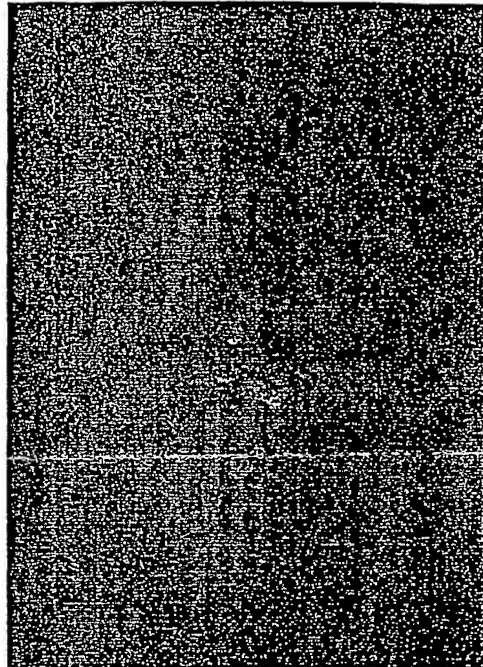


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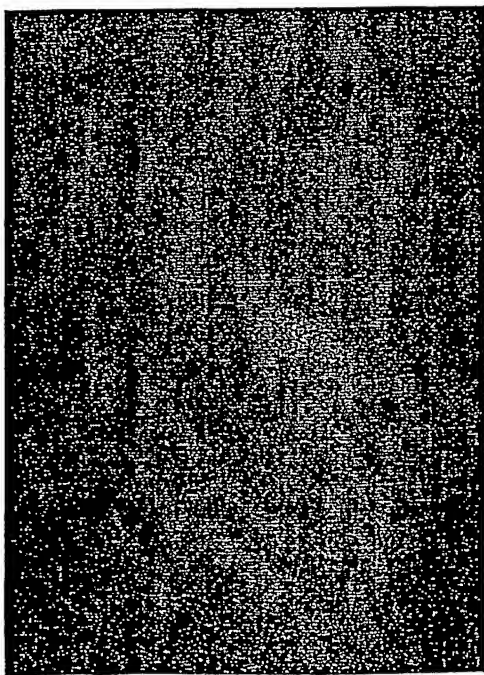
B



D



A



C

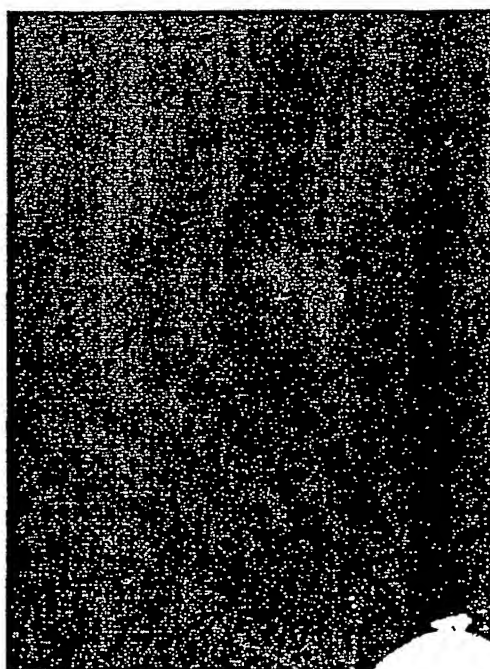
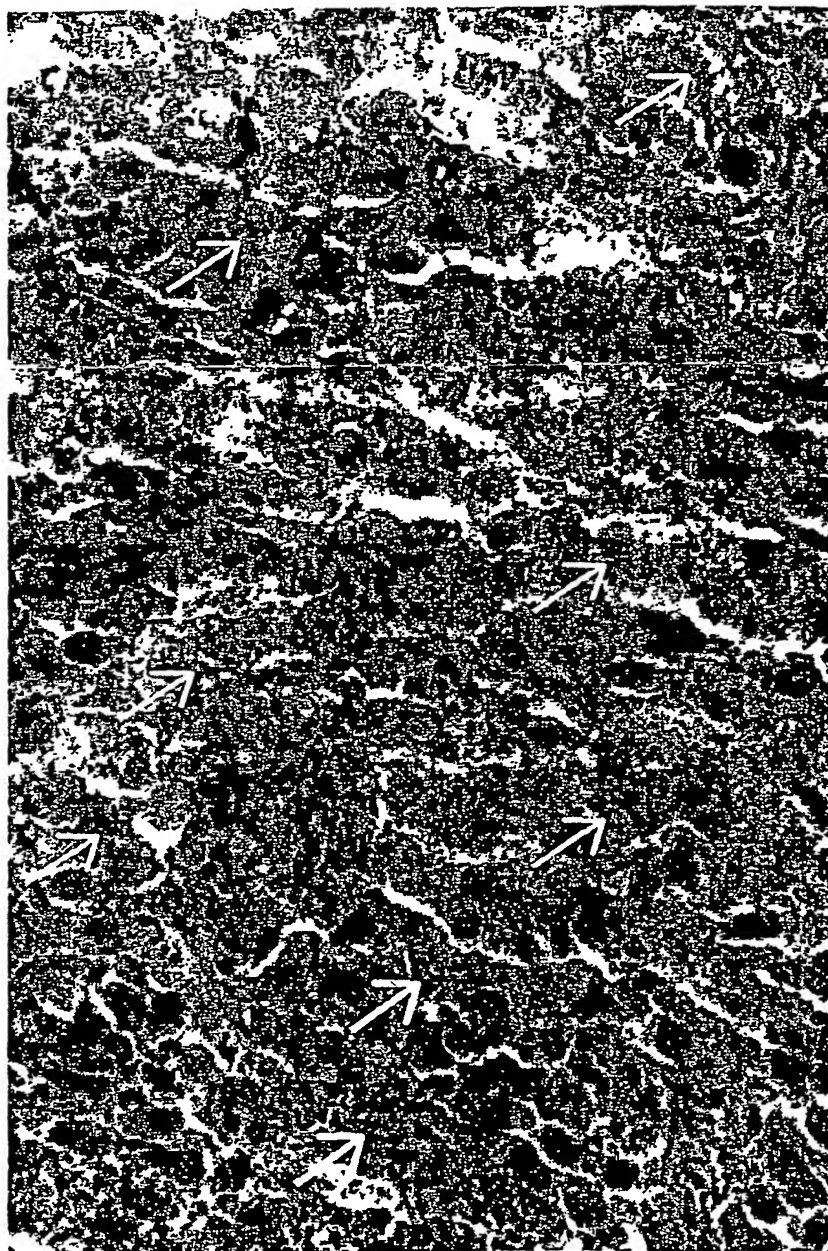
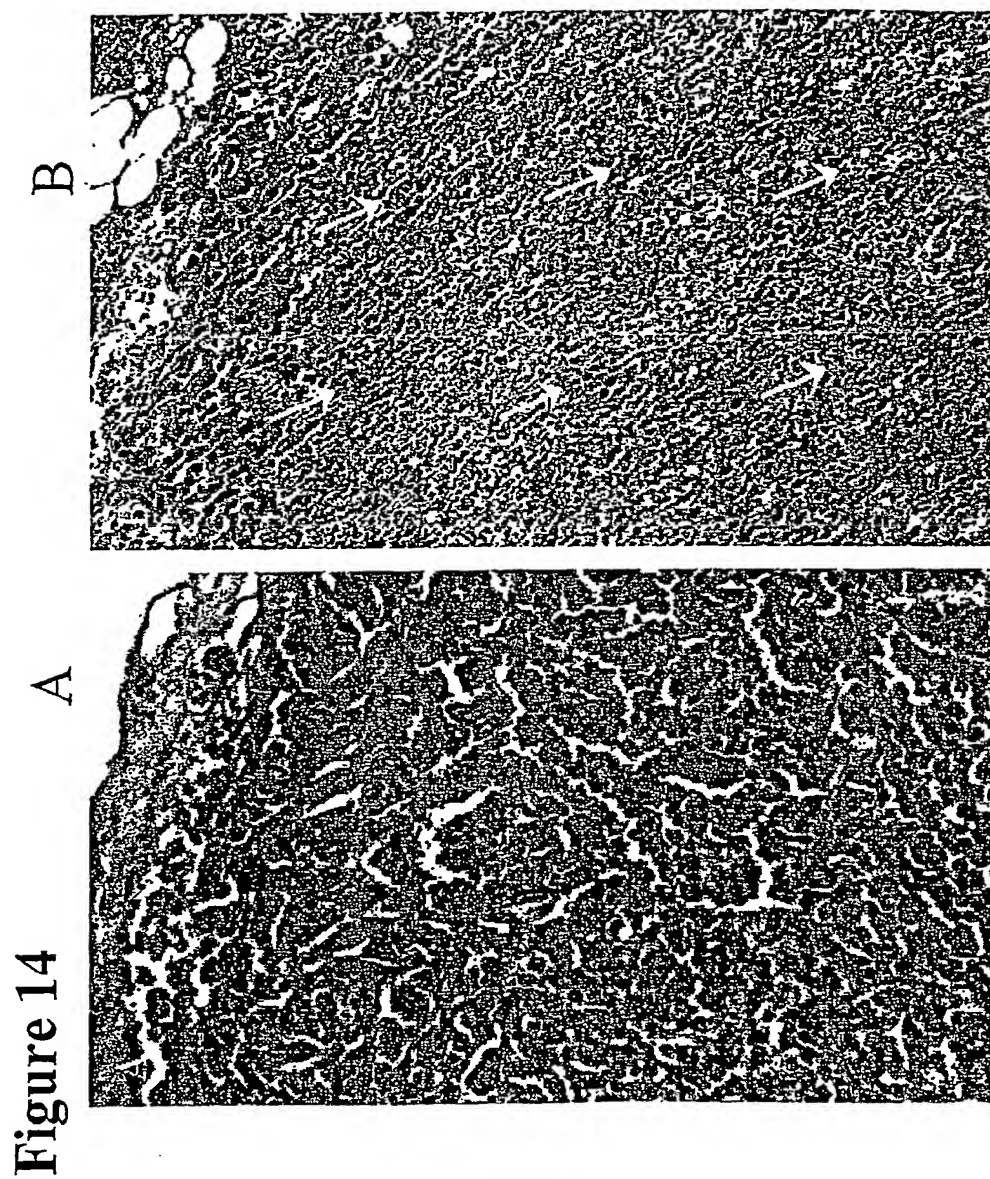


Figure 12

Figure 13





## INTERNATIONAL SEARCH REPORT

In International Application No

PCT/US 02/21812

A. CLASSIFICATION OF SUBJECT MATTER  
 IPC 7 A61K49/00 C12N1/21

According to International Patent Classification (IPC) or to both national classification and IPC

## B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

IPC 7 A61K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

EPO-Internal, BIOSIS

## C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	<p>MENG YANG ET AL: "WHOLE-BODY OPTICAL IMAGING OF GREEN FLUORESCENT PROTEIN-EXPRESSING TUMORS AND METASTASES" PROCEEDINGS OF THE NATIONAL ACADEMY OF SCIENCES OF USA, NATIONAL ACADEMY OF SCIENCE. WASHINGTON, US, vol. 97, no. 3, 1 February 2000 (2000-02-01); pages 1206-1211, XP000904536 ISSN: 0027-8424 cited in the application abstract</p> <p style="text-align: center;">---</p> <p style="text-align: center;">-/-</p>	1-22, 34, 35

☒ Further documents are listed in the continuation of box C.

☒ Patent family members are listed in annex.

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Date of the actual completion of the international search

30 October 2002

Date of mailing of the international search report

15/11/2002

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Luis Alves, D

## INTERNATIONAL SEARCH REPORT

In tional Application No  
PCT/US 02/21812

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT		
Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	<p>YANG MENG ET AL: "Visualizing gene expression by whole-body fluorescence imaging"</p> <p>PROCEEDINGS OF THE NATIONAL ACADEMY OF SCIENCES OF USA, NATIONAL ACADEMY OF SCIENCE. WASHINGTON, US, vol. 97, no. 22, 24 October 2000 (2000-10-24), pages 12278-12282, XP002210069</p> <p>ISSN: 0027-8424</p> <p>abstract</p>	16,17, 19-22, 34,35
X	<p>WO 01 25399 A (SLOAN KETTERING INST CANCER ;VION PHARMACEUTICALS INC (US))</p> <p>12 April 2001 (2001-04-12)</p> <p>abstract</p> <p>page 17, line 3 -page 19, line 7</p> <p>page 34, line 20 -page 45, line 31;</p> <p>example 6.9</p>	1-35
X	<p>WO 01 05229 A (BRIGHAM &amp; WOMENS HOSPITAL ;CHIOCCA E ANTONIO (US); GEN HOSPITAL CO)</p> <p>25 January 2001 (2001-01-25)</p> <p>page 8, last paragraph -page 12, line 10;</p> <p>claims; example 1</p>	23-27, 29,31, 33-35
X	<p>US 6 251 384 B1 (CHISHIMA TAKASHI ET AL)</p> <p>26 June 2001 (2001-06-26)</p> <p>cited in the application</p> <p>column 3, line 1.- line 47; claims;</p> <p>example 8</p>	1-22,34, 35
X	<p>WO 01 18195 A (XENOGEN CORP)</p> <p>15 March 2001 (2001-03-15)</p> <p>page 28, line 29 -page 29, line 33;</p> <p>examples 6,7</p>	1-22
P,X	<p>ZHAO MING ET AL: "Spatial-temporal imaging of bacterial infection and antibiotic response in intact animals."</p> <p>PROCEEDINGS OF THE NATIONAL ACADEMY OF SCIENCES OF THE UNITED STATES, vol. 98, no. 17, 14 August 2001 (2001-08-14), pages 9814-9818, XP002218906</p> <p>August 14, 2001</p> <p>ISSN: 0027-8424</p> <p>the whole document</p>	1-22,34, 35
P,X	<p>WO 01 71009 A (ANTICANCER INC)</p> <p>27 September 2001 (2001-09-27)</p> <p>claims; example 1</p>	1-35

# INTERNATIONAL SEARCH REPORT

International application No.  
PCT/US 02/21812

## Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This International Search Report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☒ Claims Nos.:  
because they relate to subject matter not required to be searched by this Authority, namely:  

Although claims 23 to 33 are directed to a method of treatment of the human/animal body, the search has been carried out and based on the alleged effects of the compound/composition.
2. ☐ Claims Nos.:  
because they relate to parts of the International Application that do not comply with the prescribed requirements to such an extent that no meaningful International Search can be carried out, specifically:
3. ☐ Claims Nos.:  
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

## Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

1. ☐ As all required additional search fees were timely paid by the applicant, this International Search Report covers all searchable claims.
2. ☐ As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.
3. ☐ As only some of the required additional search fees were timely paid by the applicant, this International Search Report covers only those claims for which fees were paid, specifically claims Nos.:
4. ☐ No required additional search fees were timely paid by the applicant. Consequently, this International Search Report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

### Remark on Protest

- ☐ The additional search fees were accompanied by the applicant's protest.
- ☐ No protest accompanied the payment of additional search fees.

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